GENETICS OF THE YEAST *Phaffia rhodozyma*

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Proefschrift ter verkrijging van de graad van doctor
op gezag van de rector magnificus van de Landbouwuniversiteit, Wageningen, dr. C. M. Karssen,
in het openbaar te verdedigen op vrijdag 24 oktober 1997 des namiddags te half twee in de Aula.

ISBN: 90-5485-779-X
The research described in this thesis was financed by Gist-brocades, Delft, The Netherlands.
Design: M.P. van Poorten

Stellingen behorende bij het proefschrift getiteld:
"Genetics of the yeast *Phaffia rhodozyma*", door Jan Wery. Wageningen, vrijdag 24 oktober 1997.

1. De bewering van Chun et al. (1992) dat *Phaffia* per cel bijna 4 maal zoveel DNA bevat als *Saccharomyces cerevisiae*, is niet in overeenstemming met de analyse van het chromosomenpatroon van beide gisten (dit proefschrift).
CHUN, S. B., J. E. CHIN, S. BAI, AND G-H. AN. 1992. Strain improvement of *Phaffia rhodozyma* by protoplast fusion. FEMS MICROBIOL. LETT. 93:221-226.

2. Het kritiekloos overnemen van voornoemde bewering door Cifuentes heeft vervolgens geleid tot de misvatting dat *Phaffia* diploid is.
CIFUENTES, Antonie van Leeuwenhoek, in press.

3. De wijze waarop *Phaffia* sporen vormt, hoe opwindend deze ook zijn moge, wordt door Golubev (1995) ten onrechte sexuele reproductie genoemd.
GOLUBEV, W. I. 1995. Perfect state of *Rhodomyces dendrorhous (Phaffia rhodozyma)*. YEAST 11:101-110

4. In tegenstelling tot hetgeen door Lopes et al. (1991) wordt aangenomen voor *Saccharomyces cerevisiae*, is selectiedruk wellicht niet de drijvende kracht voor plasmide-amplificatie in het ribosomale DNA van *Phaffia*.
LOPES. T. S., G-J. A. J. HAKKAART, B. L. KOERTS, H. A. RAUÉ, AND R. J. PLANTA 1991. Mechanism of high-copy-number integration of pMIRY-type vectors into the ribosomal DNA of *Saccharomyces cerevisiae*. GENE 105:83-90.

5. Het geld gestoken in het zoeken naar leven op Mars, zou effectiever besteed kunnen worden aan onderzoek tot behoud van leven op Aarde.

6. Wie na twee jaar zijn/haar AIO-schap nog als hobby ziet, heeft of geluk gehad of is gek geworden.

7. Door het streven van vakgroepen attractief te blijven voor studenten, worden de laatsten vaak getracteerd op cijfers, die niet in overeenstemming zijn met hun prestaties.

8. De wijze heeft geen onwrikbare beginselen, hij past zich aan bij anderen.
(LAO-TSE)

9. Het succes van "Paars" is te danken aan het laten varen van principes.

10. Dankzij het onderwijs zijn er minder analfabeten en meer idioten.
(ALBERT GUINON)

11. De enige ernstige overtuiging die een mens dient te hebben, is dat er niets te ernstig moet worden genomen.
(SAMUEL BUTLER)

12. Argumenten uit een mooie mond zijn onweerlegbaar.
(JOSEPH EDISON)

Contents

1.
Phaffia rhodozyma: an unconventional yeast

Phaffia rhodozyma is a basidiomycetous yeast, that grows both aerobically and anaerobically with a rather low optimum growth temperature of 21°C. The most distinctive ultrastructural feature of this yeast is the orange-red color that differs strongly from the color of other red yeasts, like *Rhodotorula* and *Rhodosporidium*, as a result of the production of the unique xanthophyll astaxanthin (Phaff et al. 1972).

Since its isolation and description in the seventies (Phaff et al. 1972; Miller et al. 1976) the unique astaxanthin producing properties of *Phaffia* have led to speculations concerning its use as an attractive alternative for the chemical production of this commercially important fish-feed ingredient. As a result many studies on *Phaffia* were concerned with the physiology of astaxanthin production and finding ways for improvement.

The academic interest in *Phaffia*, however, is in particular aroused by differences with the well characterized ascomycetous yeasts on the phylogenetical, physiological (CHAPTER 1), and genetic level (CHAPTER 2 AND 3).

The natural habitat of *Phaffia* is on slime fluxes of deciduous trees at high altitudes and is confined to high geographical latitudes. In the past century *Phaffia* was probably isolated several times by different researchers. The first publication concerning this yeast by Ludwig dates from 1891, describing the abundant presence of a particular red micro-organism in the sap of trees, he named *Rhodomyces dendrorhous*.

Several strains of the yeast were isolated after by Phaff and coworkers in 1967 and 1968 and they proposed the name *Rhodozyma montanea* (red sap of the mountains) but it was until 1976 when Miller et al. extensively described physiological and biochemical properties, including a Latin description, and named it *Phaffia rhodozyma*.

As a sexual life cycle could not be demonstrated, classification of *Phaffia* was based on ultra structural and biochemical properties. *Phaffia* was accommodated in the basidiomycetous yeasts (Miller et al., 1976) based on the ability to synthesize carotenoids, the presence of a multilayered cell wall and an enteroblastic way of budding. Furthermore, a number of differences with other (basidiomycetous) yeasts, like *Rhodotorula*, *Rhodosporidium* and *Cryptococcus*, were found, necessitating the establishment of a separate genus. (Kreger-van Rij 1987, for a survey of the classification of yeasts)

Since then, additional physiological and biochemical analyses of *Phaffia* led to different views regarding the classification of the yeast.

According to Miller et al. (1976) *Phaffia* could not be accommodated in the non-fermenting large genera of the basidiomycetous yeasts *Rhodotorula* and *Cryptococcus*, because of its ability to ferment sugars. This separation was partly supported by the finding that xylose is present in the cell wall of *Phaffia* (Sugiyama et al., 1985), which is absent in *Rhodotorula*. However, it also added to the many characteristics that *Phaffia* shares with *Cryptococcus*. Furthermore it was questioned whether gaseous fermentation, now being the sole feature that divided *Phaffia* from *Cryptococcus*, was a valuable distinguishing feature. *Phaffia* became synonymous with *Cryptococcus*. (Weijman et al., 1988),

In addition to the more traditional physiological and biochemical classification criteria the use of DNA/RNA homology studies is increasingly used as an additional tool to estimate genetic relatedness among yeasts. Especially the use of ribosomal RNA sequences (coding for 5.8S, 18S and 26S rRNA's) is extensive since these molecules are

well conserved, share a common evolutionary origin and can be readily isolated. Furthermore, so-called fingerprint regions of these molecules are sufficiently conserved, in that strains of the same species do not differ significantly in nucleotide sequence, whereas the variability in these rRNA regions is adequate to discriminate between species.

The use of rRNA sequence homology studies indicated that *Phaffia* should be retained as a separate genus. Yamada et al. (1990) found significant differences in the fingerprint region of both a 168 bp partial sequence of 18S rRNA and 150 bp partial sequence of the 26S rRNA between the genera of *Phaffia* and *Cryptococcus*.

In 1995 Fell et al. further investigated relationships of basidiomycetous yeasts at the species level by comparison of the RNA sequences of the variable B region (starting at position 368 in *Saccharomyces cerevisiae*) of the 26 S rRNA. This method was found to be adequate to differentiate between species. In this study it was found that *Phaffia* was related to the genus *Cystofilobasidium*. This genus forms secondary structures (basidia) related to the sexual state (= teleomorphic state), produces teliospores (= thick walled spores) and contains xylose in the cell walls.

Recently a stage in the life cycle of *Phaffia* was demonstrated by Golubev (1995) that is rather unconventional. Whereas in most basidiosporogenous yeasts the sexual life cycle is initiated by the mating of compatible strains followed by the formation of dikaryotic mycelium, in *Phaffia*, under certain conditions, conjugation between a mother cell and a bud triggers the formation of a slender holobasidium with apical, thin walled spores. The formation of spores by self sporulating mechanisms have been described in the telio spore-forming basidiomycetous teleomorphic yeasts. Here from a single cell a mononuclear mycelium is formed with teliospores. However, in *Phaffia* no mycelium is formed and the conjugation between a mother cell and a bud (pedogamy) with subsequent holobasidium formation prior to sporulation is an unknown phenomenon. Therefore Golubev proposed a new teleomorphic genus for this stage in the life cycle of *Phaffia*, *Xanthophyllomyces*.

From the foregoing it can be concluded that based on taxonomic criteria *Phaffia* is a quite unconventional yeast. It differs significantly from other (basidiomycetous) yeasts genera by both a unique combination of ultra-structural features and rRNA sequence. Apart from rRNA genes no other genes had been isolated from *Phaffia* and the genetic information was confined to karyotyping by agarose gel analysis (Nagy et al. 1994). In the following Chapters a genetic survey on *Phaffia* is described, showing significant differences with the genetically well-studied ascomycetous yeasts, with implications for the development of a genetic transformation system for this yeast.

Outline of this thesis

This thesis describes a study on the genetics of *Phaffia* and the development of a transformation system. The genetical analysis of *Phaffia* includes a characterization on the level of gene architecture and the genomic organization.

In CHAPTER 1 a historical overview is given on the classification of *Phaffia*.

In CHAPTER 2 the isolation and sequencing of the conserved *Phaffia* actin gene is described and a comparison is made with actin genes from different fungi. Both coding sequence homologies and structural features of these genes were studied to provide additional information on *Phaffia's* classification and to obtain more information of the genetics of this yeast on the gene level. CHAPTER 2 was published in "Yeast" (Wery et al., 1996).

In CHAPTER 3 the genetic characterization of *Phaffia* is extended by analysis of the genomic organization of the multiple ribosomal DNA (rDNA) genes. Comparisons with 13 different yeasts and filamentous fungi of ascomycetous and basidiomycetous origin are made frequently to illustrate *Phaffia's* position amongst these. Several aspects are studied. First the exact number of the rDNA units and their chromosomal location was investigated. Furthermore, the electrophoretic karyotype of *Phaffia* strain CBS 6938 and its ploidy were determined. This Chapter additionally describes the introduction of foreign DNA into *Phaffia* as an initial step towards an efficient genetic transformation system. CHAPTER 3 was published in "Gene" (Wery et al., 1997a).

The insight in the genetics of *Phaffia* has also provided a strategy towards the development of a genetic transformation system, which is described in CHAPTER 4. Parts of CHAPTER 4 were adapted from a publication by Verdoes et al. (1997). An extended version this Chapter will be published in a volume of the Manual of Industrial Microbiology and Biotechnology 2 (Wery et al., 1997b in press)

CHAPTER 5 is closely linked to CHAPTER 4 in that the *Phaffia* transformation system is described in more detail. The influence of electroporation parameters and different plasmids on both transformation efficiency and mode of integration was studied. Furthermore a possible unconventional manner of plasmid maintenance in the *Phaffia* genome is discussed.

CHAPTER 5 was submitted for publication.

CHAPTER 6, finally, deals with the economic importance of *Phaffia* for the production of astaxanthin. The use of astaxanthin in the fish feed industry, its beneficial properties for health and its possible future role as a food additive is evaluated together with the application of *Phaffia* as a commercially attractive producer of the colorant. In this context, the use of recombinant DNA technology for improved *Phaffia* strains is discussed.

2.

Structural and Phylogenetic Analysis of the Actin Gene from the Yeast *Phaffia rhodozyma*

Jan Wery, Mieke J. M. Dalderup, José ter Linde, Teun Boekhout and Albert J. J. van Ooyen

Summary

The gene coding for actin from *Phaffia rhodozyma* was cloned and sequenced. The *Phaffia* actin gene contains 4 intervening sequences and the predicted protein consists of 375 amino acids. The structural features of the *Phaffia* actin introns were studied and compared with actin introns from 7 fungi and yeasts with ascomycetous and basidiomycetous affinity. It was shown that the architecture of the *Phaffia* introns most resembles that of the basidiomycete *Filobasidiella neoformans* (perfect stage of *Cryptococcus neoformans*), whereas least resemblance occurs with the ascomycetous yeasts. Based on the intron structure, the ascomycetous yeasts can be accomodated in one group in that their splice site sequences are very similar and show less homology with the other fungi investigated, including *Phaffia*. It was demonstrated that the *Phaffia* actin introns cannot be spliced in *Saccharomyces cerevisiae*, which shows that the differences found in intron structure are significant. Alignment of the *Phaffia* actin gene with the actin sequences from the yeasts and fungi investigated showed a high level of homology both on the DNA level and on the protein level. Based on these alignments *Phaffia* showed highest homology with *F. neoformans* and both organisms were accommodated in the same cluster. In addition, the actin gene comparisons also supported the distant relationship of *Phaffia* with the ascomycetous yeasts. These results supported the usefulness of actin sequences for phylogenetic studies. The sequence presented here has been submitted to the EMBL data library under Accession Number X89898.

Introduction

Phaffia rhodozyma is a red yeast, that produces astaxanthin as the main carotenoid (Phaff et al., 1972). Astaxanthin is widely distributed in nature giving crustaceans, birds (flamingo's), fish (salmon, trout) and many other organisms their distinctive orange-red colour through their diet.

Phaffia is one of very few organisms known to synthesize astaxanthin. The growing economic importance of astaxanthin as a fish feed additive for the cultivation of salmon and trout has made *Phaffia* increasingly attractive as a possible profitable source.

Most studies conducted on this yeast were related to general physiological properties, related with taxonomy (Miller et al., 1976) and the physiology of astaxanthin production (Johnson and Lewis, 1979).

Based on classical ultrastructural and chemotaxonomic observations, like the ability to synthesize carotenoids, its multilayered cell wall, the enteroblastic way of budding and a positive reaction to Diazonium Blue B, *Phaffia* is accommodated in the basidiomycetous yeasts (Miller et al., 1976; Sugiyama et al., 1985). Very little is known about the genetics of *Phaffia* and till date no genes have been isolated. In order to gain more insight in this field, we have isolated and sequenced the *Phaffia* actin gene.

Actin plays a crucial role in elementary eukaryotic cellular processes like motility, cell growth regulation, cell differentiation and provides structural stability (Stossel, 1984; Pollard and Cooper, 1986; Pollard, 1990).

Apparently these functions have not allowed actins of different species from a broad range of phyla to diverge significantly, since the actin protein is highly conserved. This feature makes the actin gene useful for phylogenetic analysis, especially in combination with classical methods, that are mainly based on chemotaxonomic and ultrastructural studies. In this context actin gene analysis has been used previously for the grouping of organisms (Vandekerckhove and Weber, 1984; Fletcher et al., 1994).

In recent years molecular taxonomy of fungi (e.g. rRNA/rDNA comparisons) has been shown useful for phylogenetic studies (Hendriks et al., 1991 and 1992; Berbee and Taylor, 1992a,b; Suh and Sugiyama, 1993; Wilmotte et al., 1993; Kurtzman, 1994). It was demonstrated that ascomycete and basidiomycete genera were well-separated by comparing 18S and 26S rDNA sequences. Only few fungal actin genes have been isolated sofar and little is known about the validity of using these genes for phylogenetic studies among fungi.

This report describes the isolation and analysis of the *Phaffia* actin gene. As a first step to a better understanding of the genetics of *Phaffia*, the structure of the *Phaffia* actin gene, including the presence, location, size and sequence of introns, was studied and compared to the structure of actin genes from different fungi . In addition the expression of the intron containing part of the *Phaffia* actin gene in *S. cerevisiae* was studied.

Finally we have compared the actin exon sequences in order to validate the use of actin for phylogenetic studies among fungi.

Materials and Methods

Strains and media

Escherichia coli strains DH5α and JM 109 were used for transformation and amplification of recombinant plasmids according to standard methods (Sambrook et al. 1989).

Phaffia rhodozyma strain CBS 6938 and *Saccharomyces cerevisiae* strain 703 (*ura*3, *trp*1, *his*3) were used in various experiments. *Phaffia* was cultivated at 21°C in YPD medium containing 1% yeast extract, 2% bactopeptone, 2% glucose.

S. cerevisiae was cultivated at 30°C in YPD medium. *S. cerevisiae* transformants were primarily cultivated in appropriately supplemented minimal medium (0.67% yeast nitrogen base, 2% glucose) and on YPD plates containing 50 µg/ml Geneticin (G418). Transformation of *S. cerevisiae* was performed according to the LiAc method of Schiestl and Gietz (1989). Total RNA was isolated from exponentially growing *S. cerevisiae* transformants mainly according to Lacy and Dickson (1981).

Isolation of total *Phaffia* DNA

20 ml of YPD medium was inoculated with a single *Phaffia* colony. Cultivation took place at 21°C under vigorous shaking until OD_{600} reached 10 - 20. Cells were harvested by centrifuging for 5 minutes at 5000 x g and washed twice by resuspending in 20 ml of ESC (60 mM EDTA, 1.2 M sorbitol, 0.1 M Na_3Citrate, pH 7.0). Cells were resupended in 3 ml ESC containing 2.5 mg/ml Novozym 234™ to form protoplasts. Incubation took place

during 3 hours at 30°C after which the protoplasts were centrifuged for 5 minutes at 2000 x g. The pellet was resuspended in 10 ml $T_{50}E_{20}$ (50 mM Tris, 20 mM EDTA pH 7.5) containing 0.5% SDS. The mixture was extracted twice with an equal volume of Tris-equilibrated phenol pH 8.0, chloroform, isoamylalcohol (25:24:1) by vortexing followed by centrifugation for 5 minutes at 20.000 x g.

The upper aqueous phase was transferred to a fresh tube and 1/10 volume 3M NaAc pH 5.2 and 2 volumes of absolute ethanol were added. After vortexing, the mixture was centrifuged for 10 minutes at 20.000 x g. The pellet was dissolved in 0.5 ml $T_{10}E_1$ pH 8.0 containing 0.1 mg/ml RNase A. Finally the DNA was purified by phenol/chloroform extraction and ethanol precipitation and dissolved in 0.5 ml $T_{10}E_1$.

General DNA methods

Plasmid pTZ18R (Pharmacia) was used as a starting plasmid for all cloning steps. Insert sequences originating from fractionated total *Phaffia* DNA were isolated from 0.7% agarose gels by agarase treatment according to the supplier (Boehringer). DNA fragments obtained by PCR were purified from low melting point agarose using the Magic PCR Prep kit (Promega). DNA digestions and ligations were performed using enzymes purchased from BRL and applied according to the suppliers recommendations.

Positive colonies were generally picked up by colony lifting and hybridization using the DIG DNA Labelling and Detection Kit, non radioactive (Boehringer). For DNA hybridizations digested total *Phaffia* DNA was transferred to nitro-cellulose filter and hybridization was performed according to standard protocols (Sambrook, 1989).

Polymerase chain reaction (PCR)

Standard reactions were carried out in an automated thermal cycler (Perkin-Elmer). Conditions: 5 min. 95°C , followed by 30 repeated cycli; 2 min. 94°C, 2 min. 45°C, 3 min. 72°C. Ending by 1 cycle; 10 min. 72°C.

Oligo's used in this study;

Inverse PCR;

1. 5'-CGCCATCTTCTATAACAATACC-3'
2. 5'-GCATCAAGGAGAAGCTCTGCTA-3'

Oligo's used for generation of the 0.7 kb alcohol dehydrogenase I (*ADH1*) promoter from *S. cerevisiae* (Benetzen and Hall, 1982);

3. 5'-GTAGTCTTCA<u>TGGCCA</u>GAGCTCATCCTTTTGTTGTTTCCGGG-3'
4. 5'-**TGTATATGAGATAGTT**GATTGTATGCTTGGTATAG-3'

Oligo's used for generation of a 0.2 kb part of the 5'end of the kanamycin resistance gene (Km^R) gene;

5. 5'-**CTATCTCATATACA**ATGTGGATTGAACAAGATG-3'
6. 5'-GCGTGACTTC<u>TGGCCA</u>GCCACGATAGC-3'

Oligo's used for the generation of the intron part of the *Phaffia* actin gene fused to the 5' portion of the Km^R gene from pGB-Ph9;

7. 5'-**CTATCTCATATACA**ATGTACGTCGACATGCTTTC-3'
8. As oligo 6. Fusion PCR reactions were performed as described above, except that 2 DNA fragments with compatible ends were used as a template in equimolar amounts. (*Note:* restriction sites are underlined, overlapping sequences bold)

Synthesis of cDNA and amplification by PCR was performed essentially according to

Kawasaki et al. (1988). Oligo's used for cDNA synthesis and amplification of mRNA originating from pSEP3;

9. 5'-TCAGAACTCCAAGCTTTACAATCAACTATCTCATATACA-3'
10. 5'-GGGAATTCGCTTGGACTGAGCCTCGTC-3'

Oligo's used for cDNA synthesis and amplification of *S. cerevisiae* actin mRNA:

11. 5'-TACTGAATTAACAATGGATTC-3'
12. 5'-GAGCTTCATCACCAACGTA-3'

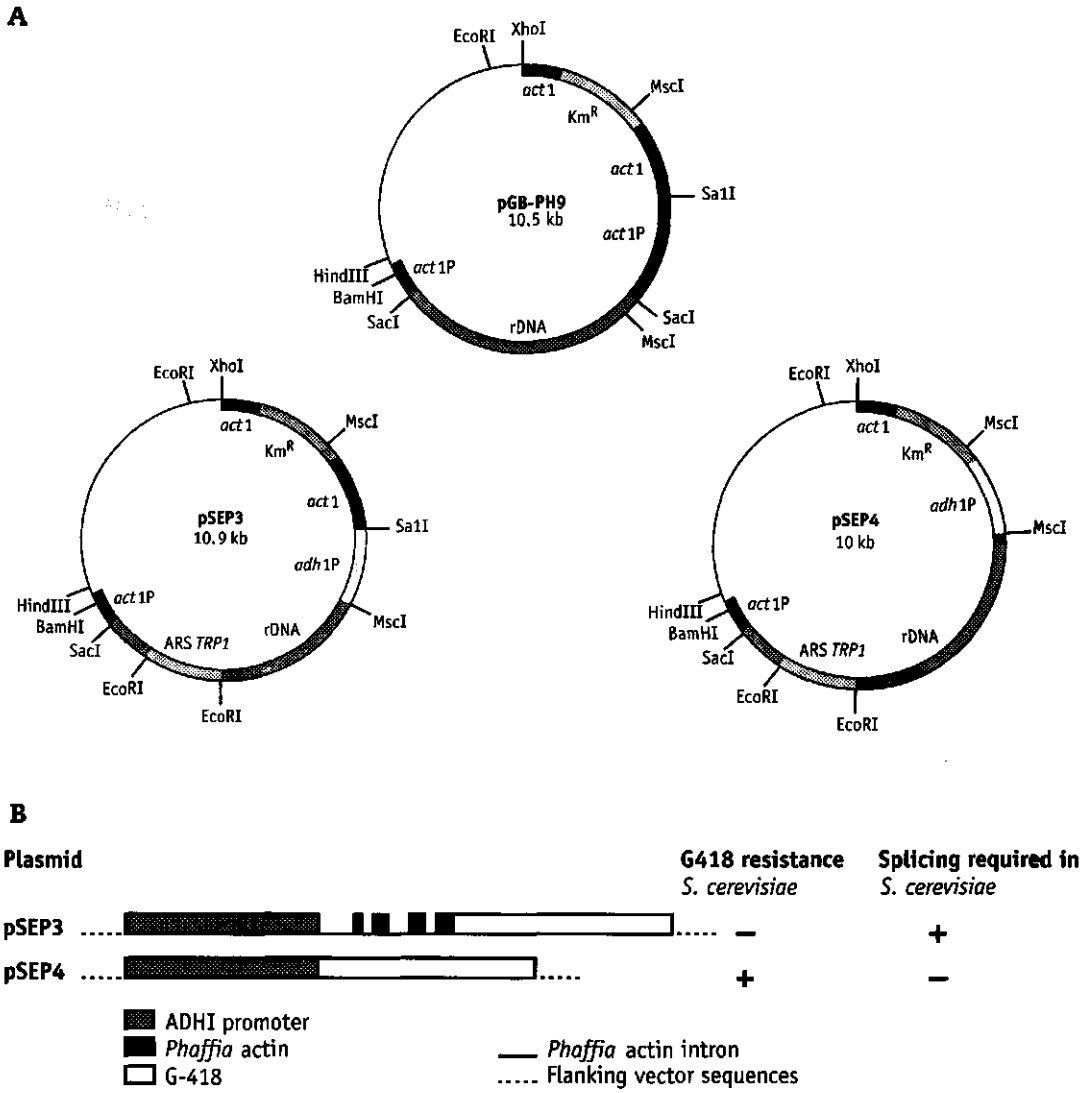

FIG. 1.

(A) Restriction maps of plasmids pGB-Ph9 (CHAPTER 3), pSEP3 and pSEP4 (as described in MATERIALS AND METHODS). *act*1 = *Phaffia* actin sequences. *act*1P = actin gene promoter. rDNA = *Phaffia* ribosomal DNA. *adh*1P = S. *cerevisiae* alcohol dehydrogenase promoter.

(B) *Phaffia* actin/Km^R fusion in pSEP3 and expression in *S. cerevisiae*.

Construction of plasmids pSEP3 and pSEP4

A 1.4 kb EcoRI fragment containing the S. *cerevisiae* ARS1TRP1 sequence (Stinchcomb et al., 1979) was inserted in the ribosomal DNA portion of pGB-Ph9 (CHAPTER 3). A MscI fragment containing the *Phaffia* actin promoter, introns and a 5' portion of the Km^R gene was deleted. Insertion of a MscI PCR product, in which the 5' portion of the Km^R gene (generated using oligo's 5 and 6) was directly fused to the S. *cerevisiae* ADH1 promoter (generated using oligo's 3 and 4), yielded pSEP4 (FIG. 1). PSEP3 was obtained by insertion of a MscI PCR fragment containing the in frame fusion between a 0.9 kb intron containing part of the *Phaffia* actin gene coding for 83 amino acids and the 5' portion of the Km^R gene (generated using oligo's 7 and 8) driven by the ADH1 promoter (generated using oligo's 3 and 4).

Phylogeny

Phylogenetic relationships were inferred from the coding sequences of the actin genes from *Phaffia rhodozyma* (this study), *Filobasidiella neoformans* (EMBL U10867), *Thermomyces lanuginosus* (EMBL X07463), *Aspergillus nidulans* (M22869), *Schizosaccharomyces pombe* (Y00447), *Saccharomyces cerevisiae* (J01310), *Kluyveromyces lactis* (M25826), *Candida albicans* (X16377) and *Absidia glauca* (M64729) using neighbour-joining with Kimura 2-parameter distances (Kumar et al., 1993). Robustness of the resulting tree was tested by bootstrap analysis using 1000 replicates. Alternatively, the sequences were analyzed using PAUP version 3.0s (Swofford, 1991).

RESULTS

Isolation of the *Phaffia* actin gene

Total *Phaffia* DNA was digested with several enzymes and separated by agarose gel electrophoresis. After blotting the DNA was hybridized using a PCR amplified 200 bp *Kluyveromyces lactis* actin DNA fragment. The *Phaffia* DNA only hybridized weakly as shown by the rather faint signals (FIG. 2).

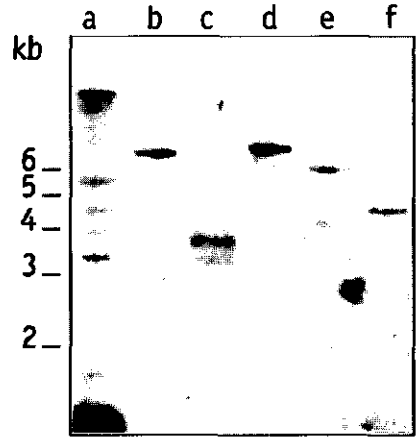

FIG. 2.
Southern blot of digested total *Phaffia* DNA, hybridized with a 200 bp *K. lactis* actin DNA fragment. Lane a contains total *K. lactis* DNA digested with *Hind*III as a positive control, and lanes b-f contain total *Phaffia* DNA digested with: b, *Eco*RI; c, *Sal*I/*Eco*RI; d, *Sal*I; e, *Bgl*II; f, *Eco*RI/*Bgl*II.

A 3.8 kb EcoRI-SalI *Phaffia* DNA fragment, hybridizing with the *K. lactis* actin probe, was cloned in pTZ18R. The resulting plasmid PGB-Ph1 was partly sequenced and based on sequence comparison with the *K. lactis* and *S. cerevisiae* actin genes (Deshler et al., 1989; Gallwitz and Sures, 1980) the 5' end of the gene including the promoter region was missing.

The restriction sites around the *Phaffia* actin gene were mapped by digestion of chromosomal DNA with several enzymes followed by hybridization with a 500 bp SalI-BamHI fragment at the 5' end of *Phaffia* actin gene insert from pGB-Ph1 (not shown). A 3.1 kb hybridizing fragment was detected using XhoI. Since it was known that a 1.4 kb SalI-XhoI portion is located at the 5' end of the actin insert in pGB-Ph1, it was concluded that the hybridizing band contained a 1.7 kb sequence flanking the 5' end of the *Phaffia* actin insert in pGB-Ph1.

This 1.7 kb sequence was isolated using the inverse PCR technique (Ochman et al., 1988). Chromosomal DNA from *Phaffia* was digested with XhoI and subsequently ligated. PCR was performed with the ligated material using oligo's 1 and 2 (MATERIALS AND METHODS), that were designed on the basis of known sequences 60 bp downstream the SalI-site and 60 bp upstream the XhoI-site. A PCR product with an expected length of 1.7 kb was obtained.

After digestion with SalI and XhoI the PCR fragment was cloned in the SalI site of pTZ18R, yielding pGB-Ph2. A 0.6 kb 3' portion of the *Phaffia* DNA insert in pGB-Ph2 was sequenced and found to contain the 5' end of the *Phaffia* actin gene including the putative ATG start codon. Thus the full length *Phaffia* actin gene and promoter were obtained.

The architecture of *Phaffia* actin introns and comparison with other fungal actin introns

The 1808 bp *Phaffia* actin gene codes for 375 amino acids (FIG. 3). The 5' part of the gene contains 4 introns, ranging in length from 70 to 337 bp. Sequence elements that play an important role in the splicing process are partly conserved.

In TABLE 1 the 5' splice sites, the branch sequences and the 3' splice sites are depicted. The 5' splice site, consisting of 9 nucleotides, includes 3 nucleotides of the exon adjacent to 6 nucleotides of the 5' part of the intron. Exon intron boundaries are indicated by a slash. If compared with the consensus 5' splice sequence CAG/GTAAGT (Jacob and Gallinaro, 1989), the 5' splice sites of the *Phaffia* actin introns display 5 to 7 complementary matches. In all four introns the first and the last GT in the intron part of the 5' splice site are conserved.

In fungal introns the sequence NPuCTPuAC has been proposed as the consensus branch sequence (Rambosek and Leach, 1987). In this sequence the last A is considered to be important in the splicing process in that it is involved in lariat formation by covalent binding to the first G of the intron (Domdey et al., 1984; Padgett et al., 1984). Based on this criterion putative branch sites can be proposed within all four *Phaffia* actin introns. In these branch sequences 4 to 6 nts match with the consensus sequence. In all four branch sites the first and fourth T in the sequence as well as the important last A are conserved.

The 3' splice site (PyAG) is completely conserved in all 4 *Phaffia* actin introns. The distance between the putative branch sites and the 3' splice site ranges from 7-67 bp. In mammalian introns this spacing varies from 10-40 bp (Green, 1986; Padgett et al., 1986), in *S. cerevisiae* from 3-134 bp (Gallwitz et al., 1987; Woolford, 1989) and in most of the in-

tron containing non actin genes in *Schizosaccharomyces pombe* from 3-16 bp (Prabhala et al., 1992). Similar to mammalian and *S. pombe* introns (Green, 1986; Padgett et al., 1986; Prabhala et al., 1992), Py stretches can be assigned in this region in the first 2 introns of the *Phaffia* actin gene. Considering the complete intron sequences, except for the third intron, they all show high Py content (64-68%).

TABLE 1. Consensus actin intron sequences

Intron		size (bp)	pos.	5'	Putative Branch		3'	Group
Phaffia	1	338	3	CAT/GTACGT	TATTGAC	67	TAG/GGA	1
	2	98	363	CTG/GTATGT	TTCTTAT	7	TAG/GTG	
	3	177	532	CCC/GTAAGT	TGCTGAT	34	CAG/CTC	
	4	71	778	TGG/GTTCGT	TATTAAC	18	CAG/TGA	
F. neoformans	1	52	12	AAG/GTACGT	AGCTAAC	7	CAG/TGC	1
	2	45	145	CCC/GTAAGT	AACTCAC	5	CAG/TTC	
	3	47	258	CGG/GTATGT	TATTAAC	6	CAG/TGA	
	4	52	1068	TTG/GTAAGT	ACGTAAC	7	TAG/CCG	
	5	52	1260	GGA/GTAAGT	CGCTAAC	8	TAG/TCG	
T. lanuginosus	1	148	7	AAG/GTAAGC	TGCTAAT	15	CAG/AGG	1
	2	157	187	TGG/GTATGT	AGCTGAC	10	CAG/TTC	
	3	146	401	TCC/GTAAGT	TACTGAC	7	TAG/CGT	
	4	58	579	TGG/GTAATT	CTCTAAC	6	TAG/TAT	
	5	57	1410	ATG/GTAGGT	CACTAAC	7	TAG/TCT	
A. nidulans	1	111	7	AAG/GTAAGG	CGCTAAT	21	CAG/AGG	1
	2	232	150	TGG/GTATGT	TGCTGAC	11	TAG/TTC	
	3	194	439	TCC/GTAAGT	TGCTAAC	9	CAG/CCT	
	4	59	665	TGG/GTAAAT	CGCTGAC	8	TAG/TAT	
	5	56	1497	ATG/GTATGT	CACTAAC	7	TAG/TCT	
	6	54	1786	AAG/GTATGA	GTCTAAT	7	CAG/CTC	
S. bayanus	1	348	10	CTG/GTATGT	TACTAAC	29	TAG/AAG	2
S. cerevisiae	1	304	10	CTG/GTATGT	TACTAAC	39	TAG/AGG	2
K. lactis	1	777	7	CTG/GTATGT	TACTAAC	118	CAG/AGG	2
C. albicans	1	656	9	GTG/GTATGT	TACTAAC	9	TAG/AAG	2
U1SnRNA*				CAG/GTAAGT				
U2SnRNA					TACTAAC			
Consensus†				GTPuNGT	PuCTuAC		PyAG	

Pos. = nucleotide position of the intron within the gene. 5' = 5' splice sequence. Branch = branch sequence. 3' = 3' splice sequence. Exon intron boundaries are indicated by a slash. The number between the branch sequence and the 3' splice sites indicates the distance in nucleotides.

*Jacob and Gallinaro, 1989

† Rambosek and Leach,1987

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TCAACACAAC CGTCCGTGCC CAGGCCCCAT CTCTTTATTT CCCTCCTCTT TCTCTTCTTC
CTTCCTTCTT CCCGACCACC ACTCACTCTT ACTCTCTCTT TCTCTCAAAA CAAAACTCCC
GTACCTCCTC CACCTTTAAA AAGAAACAGT CTAGCCCACC   AT  GTACGTCGAC
                                               Met
                                               1
ATGCTTTCTT TCTCTTTCTG AAGTGTATGC GTGTGGTATT GTTATAGAAG ATGGCGATCG
GAAAGGCTCA TCGCCTCCTT TTTCTTTTTC ACTTCATCTG CGTTTCGCCT CTTTTTTTTT
TAAATCATCA TTTCTTCGTC TTTTTTGACA CTTGATTGTG CACTGCCCCT TTCTTTTTCT
CTTGCTTACG TCTTTCTCCT TCCCCGTCTT TGGATTTACC TCGGCCATCT TATAATCAAT
TCACTCTACC CTATTGACTG CGGCCTTATC ATCCATCCTT TTTTTTCCAT ATCGTGTGAT
GGATATGCGA TGGATTCTTC AACTCTAG G GAT GAT GAA GTT GCC GCC CTC
                                  Asp Asp Glu Val Ala Ala Leu
                                                  5
GTATGTGTTT CTAAATATCT TCATGATGCG AATTGGCTCC TTGGCTCATA TCCGCTTTCT
TCGTTGTCTC TTGTAATGGT TTTCTTATCA TTCATTAG GTG ATT GAT AAC GGA
                                              Val Ile Asp Asn Gly
                                                    10
TCC GGA ATG TGC AAG GCC GGA TTC GCC GGA GAT GAT GCT CCC CGA GCC
Ser Gly Met Cys Lys Ala Gly Phe Ala Gly Asp Asp Ala Pro Arg Ala
     15                  20                  25
GTC TTC  CC  GTAAGTACTA GTATCGTTTC GTCGAGCTTG GTTAAATTCA
Val Phe  Pro
 30
TGACAGAGCA AAAGCGATCC AAGAACATGC TTCACGTCTC AGTCTTGATA TTCGTAAAGA
CGGAGTGGCA ACTCCTTGTA TGGATGACGC AACTGCTGAT CGTACCTCTT TCTGAATTGG
TTAACCAATC TTCACAG C TCC ATT GTT GGA CGA CCC CGA CAC CAG GGT GTT
                    Ser Ile Val Gly Arg Pro Arg His Gln Gly Val
                                35                  40
ATG GTC GGT ATG GGA CAG AAG GAC TCC TAC GTT  GG  GTTCGTATCT
Met Val Gly Met Gly Gln Lys Asp Ser Tyr Val  Gly
     45                  50                  55
TTCACATCTC TTGATGTCGT AACCGGCTCT TGTTATTAAC CTGATGTCTT CTATGCGGCA
G T GAC GAG GCT CAG TCC AAG CGA GGT ATT CTT ACC CTC AAG TAC CCT
    Asp Glu Ala Gln Ser Lys Arg Gly Ile Leu Thr Leu Lys Tyr Pro
                    60                  65                  70
ATC GAG CAC GGA ATC GTC ACC AAT TGG GAC GAT ATG GAG AAG ATC TGG
Ile Glu His Gly Ile Val Thr Asn Trp Asp Asp Met Glu Lys Ile Trp
                75                  80                  85
CAC CAC ACC TTT TAC AAC GAG CTT CGA GTT GCC CCT GAG GAG CAC CCC
His His Thr Phe Tyr Asn Glu Leu Arg Val Ala Pro Glu Glu His Pro
            90                  95                  100
GTC CTT CTT ACT GAG GCT CCT CTA AAC CCC AAG GCT AAC AGA GAG AAG
Val Leu Leu Thr Glu Ala Pro Leu Asn Pro Lys Ala Asn Arg Glu Lys
        105                 110                 115
ATG ACC CAG ATC ATG TTC GAG ACC TTC AAC GCT CCC GCT TTC TAC GTT
Met Thr Gln Ile Met Phe Glu Thr Phe Asn Ala Pro Ala Phe Tyr Val
    120                 125                 130
GCC ATT CAG GCC GTG CTT TCT TTG TAC GCC TCT GGT CGA ACC ACC GGT
```

FIG. 3.

```
Ala Ile Gln Ala Val Leu Ser Leu Tyr Ala Ser Gly Arg Thr Thr Gly
135                 140                 145                 150
ATC GTG CTC GAC TCT GGA GAC GGA GTC AGT CAC ACT GTT CCT ATC TAC
Ile Val Leu Asp Ser Gly Asp Gly Val Ser His Thr Val Pro Ile Tyr
                155                 160                 165
GAG GGT TTC GCC CTT CCC CAC GCC ATC CTC CGA TTG GAC TTG GCC GGT
Glu Gly Phe Ala Leu Pro His Ala Ile Leu Arg Leu Asp Leu Ala Gly
            170                 175                 180
CGA GAC TTG ACC GGG TAC CTT GTC AAG TGC TTG ATG GAG CGA GGA TAC
Arg Asp Leu Thr Gly Tyr Leu Val Lys Cys Leu Met Glu Arg Gly Tyr
        185                 190                 195
CCT TTC ACC ACC ACT GCC GAG CGA GAG ATT GTT CGA GAC ATC AAG GAG
Pro Phe Thr Thr Thr Ala Glu Arg Glu Ile Val Arg Asp Ile Lys Glu
    200                 205                 210
AAG CTC TGC TAC GTA GCT CTC GAT TTC GAG CAG GAG ATG CAG ACC GCC
Lys Leu Cys Tyr Val Ala Leu Asp Phe Glu Gln Glu Met Gln Thr Ala
215                 220                 225                 230
GCT CAG TCT TCC CAG CTC GAG AAG TCG TAC GAG CTT CCC GAC GGA CAG
Ala Gln Ser Ser Gln Leu Glu Lys Ser Tyr Glu Leu Pro Asp Gly Gln
                235                 240                 245
GTT ATC ACC ATT GGA AAC GAG CGA TTC CGA TGC CCT GAA GCT CTC TTC
Val Ile Thr Ile Gly Asn Glu Arg Phe Arg Cys Pro Glu Ala Leu Phe
            250                 255                 260
CAG CCC TCT TTC CTC GGA CTC GAG GCC GCC GGT ATT CAC GAG ACC ACC
Gln Pro Ser Phe Leu Gly Leu Glu Ala Ala Gly Ile His Glu Thr Thr
        265                 270                 275
TAC AAC TCG ATC ATG AAG TGT GAT CTT GAT ATC CGA AAG GAT CTC TAC
Tyr Asn Ser Ile Met Lys Cys Asp Leu Asp Ile Arg Lys Asp Leu Tyr
    280                 285                 290
GGA AAC GTC GTC CTT TCC GGA GGA ACC ACC ATG TAC TCT GGT ATT GCC
Gly Asn Val Val Leu Ser Gly Gly Thr Thr Met Tyr Ser Gly Ile Ala
295                 300                 305                 310
GAT CGA ATG CAG AAG GAG ATT ACT TCC CTT GCC CCG TCG TCG ATG AAG
Asp Arg Met Gln Lys Glu Ile Thr Ser Leu Ala Pro Ser Ser Met Lys
                315                 320                 325
GTC AAG ATT GTT GCT CCT CCT GAG AGG AAG TAC TCC GTC TGG ATT GGA
Val Lys Ile Val Ala Pro Pro Glu Arg Lys Tyr Ser Val Trp Ile Gly
            330                 335                 340
GGA TCC ATC TTG GCT TCC CTC AGC ACT TTC CAA TCT ATG TGG ATC TCA
Gly Ser Ile Leu Ala Ser Leu Ser Thr Phe Gln Ser Met Trp Ile Ser
        345                 350                 355
AAG CAG GAG TAC GAC GAG GCT GGA CCT TCC ATC GTC CAC CGA AAG TGC
Lys Gln Glu Tyr Asp Glu Ala Gly Pro Ser Ile Val His Arg Lys Cys
    360                 365                 370
TTC TAAGTCAACA AAGTCTTTCT ATCCTTTAAG GCAGAACGGT TTCTTTTCTT
Phe
375
```

FIG. 3.

Nucleotide sequence of the *Phaffia* actin gene. The amino acid sequences of exons 1 to 5 are shown below their respective DNA sequences. The splice sequences are underlined.

The *Phaffia* actin intron architecture was compared to the actin introns of various ascomycete and basidiomycete fungi, including *Saccharomyces bayanus*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Candida albicans*, *Filobasidiella neoformans*, *Thermomyces lanuginosus* and *Aspergillus nidulans*. The actin sequences were collected from the EMBL/GenBank database. Based on this comparison the investigated species are divided into two groups. Group 1 accommodates the filamentous fungi *T. lanuginosus* and *A. nidulans*, and the basidiomycetous yeasts *F. neoformans* (the perfect stage of *Cryptococcus neoformans*) and *P. rhodozyma*.

Accommodated in group 2 are the ascomycetous yeasts *S. cerevisiae*, *S. bayanus*, *K. lactis* and *C. albicans*. In group 2 the actin genes contain only 1 intron, whereas group 1 actins contain 4 to 6 introns. There is also a size difference. Group 2 introns, ranging in size from 304-777 bp are on the average significantly longer than the 52-337 bp group 1 introns.

Furthermore large differences exist between the consensus sequence elements involved in splicing. In group 2 all 5' splice sites conform to the consensus sequence $^{C}/_{G}$TG/GTAGT. The branch sequences in group 2 introns are all in accordance with the sequence TACTAAC. Finally high homology also exists between the 3' splice sites of group 2 members. They all conform to PyAG/APuG.

In group 1 little of the homology displayed in group 2 remains. The exon part of the 5' splice sequence shows no consensus at all, whilst in the intron part only the first GT is maintained throughout the group. Other bases in the 5' splice site are more or less variable. The branch sequences (TACTAAC box) in group 1 introns also show a high degree of variability with only a well conserved second T and last A (branch point) and a Py as a last base. The intron part of the 3' splice site is also well conserved in group 1 introns, however opposite to group 2 the exon part does not comply with any consensus.

On the level of splice site consensus *P. rhodozyma* and *F. neoformans* share a number of similarities, that do not exist among the other members of group 1.

Phaffia intron 3 resembles *F. neoformans* intron 2 in that they both contain CCC/GTAAGT as the 5' splice site. In addition the sequences GTACGT and GTATGT occur in both organisms as the intron part of the 5' splice site. Furthermore *Phaffia* intron 4 and *F. neoformans* intron 3 contain identical branch sites (TATTAAC) and 3' splice sites (CAG/TGA).

Phaffia actin introns cannot be spliced in *S. cerevisiae*

From the foregoing it can be concluded that the *Phaffia* actin introns differ from the *S. cerevisiae* actin intron. We questioned to what extend these differences in intron architecture have influence on splicing efficiency and examined whether the *Phaffia* actin introns could be correctly spliced by *S. cerevisiae*.

Obviously at least some impairment of the splicing efficiency could be anticipated. For example, the branch site sequences of the *Phaffia* actin introns show little similarity with the *S. cerevisiae* TACTAAC box. In addition the intron part of the 5' splice sites differs up to 2 bp.

However it can be argued that there also exist similarities in the intron architecture of both organisms. First like in *S. cerevisiae*, all *Phaffia* introns are located near the 5' end of the actin gene, opposite to *A. nidulans* and *F. neoformans*. It was shown previously by Yoshimatsu and Nagawa (1994), that a 5' end positioning of introns is important for correct splicing in *S. cerevisiae*. Second the intron part of the 3' splice site is highly conserved, whilst the maximum distance between the branch site and the 3' splice site in

Phaffia is 67 bp. It was shown previously that a 66 bp extension of this spacer in the S. *cerevisiae* actin gene to 106 bp still allowed relatively efficient splicing (Cellini et al., 1986).

Finally only the first *Phaffia* actin intron exceeds the size of the actin intron in S. *cerevisiae* marginally, while the other *Phaffia* actin introns are smaller.

To investigate the possible splicing of the introns by S. *cerevisiae*, we have constructed plasmid pSEP3 and pSEP4 (FIG. 1). In pSEP3 the Km^R gene is used as a reporter gene for the correct splicing of *Phaffia* actin introns by S. *cerevisiae*. It is known that this transposon Tn5 encoded gene confers kanamycin resistance to E. coli and G418 resistance to S. *cerevisiae* (Jimenez and Davies, 1980).

Plasmid pSEP3 contains the ARS1 sequence and the TRP1 gene of S. *cerevisiae* (Stinchcomb et al., 1979). It also harbours the Km^R gene fused in frame to a 83 amino acids coding intron containing part of the *Phaffia* actin gene under control of the strong S. *cerevisiae* ADH1 promoter. Plasmid pSEP4 differs from pSEP3 in that the Km^R coding sequence is directly fused to the ADH1 promoter (FIG. 1B).

As pSEP3 and pSEP4 were constructed using PCR techniques, 3 of each plasmid, obtained from independent E. coli clones were transformed to S. *cerevisiae* giving rise to the same results.

Transformants were initially screened for a TRP1 phenotype. Transformant colonies were subsequently resuspended and dilutions containing 100-1000 cells were plated on YPD plates containing G418. Upon transformation with pSEP4, transformants were obtained that were resistant to high G418 concentrations exceeding 1 mg/ml. However pSEP3 transformants did not show any resistance at 50 µg/ml, a concentration at which adaptation is observed in non transformed cells. This result indicated that the *Phaffia* actin introns in pSEP3 were not spliced efficiently.

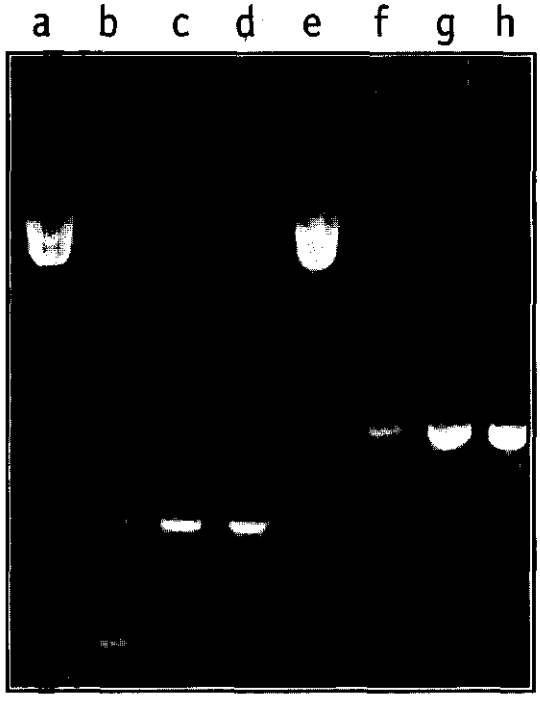

FIG. 4.
Agarose electrophoresis of PCR amplified cDNA prepared from total RNA from S. *cerevisiae* transformed with pSEP3.
Lane a, 123 bp DNA ladder.
Lanes b - d contain PCR amplified products, using primers located at each side of the S. *cerevisiae* actin gene intron, originating from: lane b, cDNA from total RNA; lane c, total RNA; lane d, total DNA. Lane e, 123 bp DNA ladder.
Lanes f - h contain PCR amplified products, using primers located at each side of the *Phaffia* actin in-tron containing part of pSEP3, originating from: lane f, cDNA from total RNA; lane g, total RNA; lane h, plasmid pSEP3.

However the event of partial splicing could not be ruled out, since some *Phaffia* actin introns share more homologies with the *S. cerevisiae* actin intron than other. Therefore it was decided to analyze the RNA of pSEP3 transformants by means of Reverse Transcriptase-PCR (Kawasaki et al., 1988). Copy DNA prepared from total RNA from a pSEP3 transformant was amplified by PCR using oligo primers that were designed on regions at both sides of the intron containing part of pSEP3. No splicing products could be detected, only a 0.9 kb PCR product representing amplified unspliced cDNA and contaminating plasmid DNA was visible (**Fig. 4, lane f**).

As a control on the procedure PCR was also performed on the cDNA using oligo's that were designed on sequences of the *S. cerevisiae* actin gene at both sides of the intron. Besides a 0.5 kb DNA fragment originating from contaminating chromosomal DNA and unspliced RNA, also a 180 bp DNA fragment was obtained, which is exactly the size of correctly spliced actin pre-mRNA (**Lane b**). From these results it can be concluded that none of the *Phaffia* actin introns can be spliced in *S. cerevisiae*.

Alignment of actin sequences shows a close phylogenetic relationship between *Phaffia* and *Cryptococcus*

It was investigated if phylogenetic studies based on actin homology were consistent with the phylogenetic background of the fungi involved. Therefore the actin sequences of *S. bayanus*, *S. cerevisiae*, *K. lactis*, *C. albicans*, *F. neoformans*, *T. lanuginosus*, *A. nidulans*, as well as *Schizosaccharomyces pombe* and the zygomycete *Absidia glauca*, that were not included in the intron architecture comparisons, were collected from the EMBL/GenBank database.

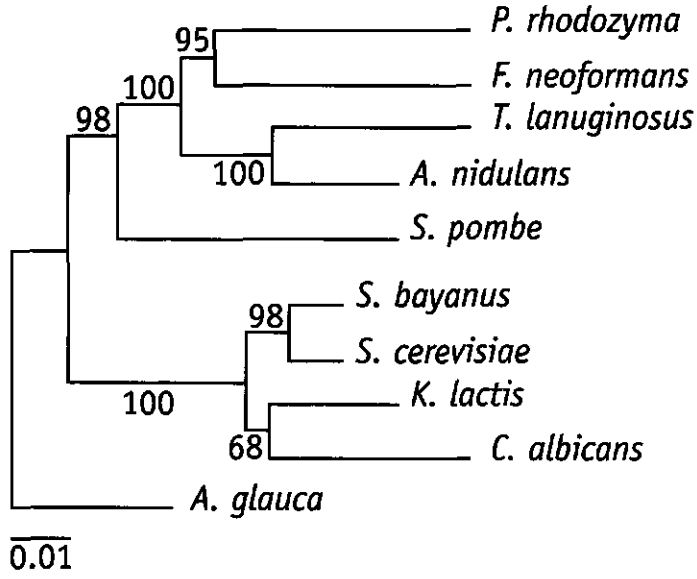

Fig. 5.
Phylogenetic tree using nucleotides of the coding regions of the actin gene of some ascomycetous and basidiomycetous fungi, including some yeasts. The tree was generated using neighbour-joining with Kimura 2-parameter distance (Kumar et al., 1993). Bootstrap values are indicated on the branches and branch lengths are indicated by the bar.

After removal of non coding stretches (e.g. introns) the nucleotide sequences were aligned and phylogenetic relationships were inferred using neighbour-joining with Kimura 2-parameter distances (Kumar et al., 1993). Robustness of the resulting tree was tested by bootstrap analysis using 1000 replicates (FIG. 5). The actin gene from the zygomycete *A. glauca* was included as an outgroup member.

The nucleotide sequence of the actin gene of *Phaffia* was found most similar to that of the other basidiomycetous yeast, *F. neoformans*. These two species formed a well supported terminal branch (bootstrap value 95%). Two filamentous ascomycetous species, *T. lanuginosus* and *A. nidulans*, forming a sister group with the afore mentioned basidiomycetes, are another well supported terminal branch. The ascomycetous budding yeasts *S. bayanus*, *S. cerevisiae*, *K. lactis* and the anamorph *C. albicans* formed a well supported cluster, with the fission yeast *S. pombe* only remotedly related. Essentially the same phylogenetic relationships were obtained using parsimony methods (data not shown).

The amino acid sequences of the protein resulted in the same phylogenetic tree (data not shown). The actin protein of *Phaffia* was found to be most similar with that of *F. neoformans*. Surprisingly, the actin of *S. pombe* displayed a very high homology to the *Phaffia* and *F. neoformans* actin proteins.

DISCUSSION

In this report the isolation, cloning and analysis of the actin gene from *Phaffia rhodozyma* is described. This is the first *Phaffia* gene to be isolated and its architecture, involving the presence of introns and their structure, was shown to be quite different from actin genes studied sofar from genetically well studied yeasts like *K. lactis*, *S. pombe* and *S. cerevisiae*. Since the phylogenetic relationships of these organisms are in agreement with the large differences in actin gene architecture, it was investigated whether this trend was also present if more actin genes from various fungi were analysed and compared.

A comparison made on the level of splice site consensus indeed supported the differences that exist between the species investigated. It was shown that the ascomycetous yeasts are clearly separated as a group from the filamentous ascomycetes and the basidiomycetous yeasts including *Phaffia* and *F. neoformans*. Moreover, the common basidiomycetous background of the latter two taxa was demonstrated by a number of similarities in splice site consensi, that were not shared with the other species.

The large phylogenetic distance between the ascomycetous yeast *S. cerevisiae* and the basidiomycete *Phaffia*, as confirmed by actin intron comparison, was emphasized by the fact that the introns of the latter were not spliced out by the first. In the past splicing of the *K. lactis* actin intron and the *Candida maltosa FDH1* intron in *S. cerevisiae* was studied (Deshler et al., 1989; Sasnauskas et al. 1992). Despite the fact these organisms belong to different genera the splice sequence element homologies were sufficient for efficient splicing. We demonstrated that none of the *Phaffia* actin introns are spliced in *S. cerevisiae*. Apparently the splice site sequences, in particular the branch sites, diverge to much. In this context it is noteworthy to mention that it was shown previously that the introns of the xylanase gene of the basidiomycetous yeast *Cryptococcus albidus* were not spliced in *S. cerevisiae* (Moreau et al., 1992). The putative branch sites in the introns of this gene (Boucher et al., 1988), also differ significantly from the *S. cerevisiae* TACTAAC consensus.

The results obtained from the actin exon alignments are in accordance with earlier findings based on rRNA/rDNA sequencing studies. It has been reported that molecular phylogenetic studies using 18S rRNA sequences divide basidiomycete and ascomycete taxa (Hendriks et al., 1991; Suh and Sugiyama, 1993; Wilmotte et al., 1993). This is supported by our actin gene comparisons. In addition, in our phylogenetic tree the ascomycetous yeasts, except *S. pombe*, form a cluster distinct from the filamentous ascomycetous fungi.

Our study suggests a distant relationship between *S. pombe* and the ascomycetous yeasts investigated. Partial and complete 18S rRNA studies demonstated that *S. pombe* is only remotely related with budding ascomycetous yeasts (Kurtzman, 1989; Sogin et al., 1989; Eriksson et al., 1993).

In conclusion, the results presented in this report show, that not only based on classical taxonomic features but also on the level of actin gene architecture, *Phaffia* differs greatly from the ascomycetous budding yeasts. This knowledge is very useful in future genetic research on this yeast. Furthermore, it was shown that actin gene comparisons among fungi are useful for phylogenetic studies.

From the results described in this chapter it can be deduced that on the gene level *Phaffia* is clearly distinct from genetically well studied yeasts like *S. cerevisiae*, *K. lactis* and *S. pombe*. Furthermore, based on comparison of both the structure of the actin gene and the DNA/protein sequence *Phaffia* is phylogenetically closer related to the filamentous fungi *A. nidulans* and *T. lanuginosus*. The same is true for *F. neoformans*.

This conclusion was supported by the finding of Verdoes et al. (1997) that in phylogenetic analyses the *Phaffia* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene formed a cluster with the *gpd* sequences of filamentous basidiomycetes. Significant lower homologies were found with the ascomycetous yeasts, like *S. cerevisiae* and *K. lactis*.

In addition it was found that comparison of codon usage of *Phaffia* in the actin and *gpd* gene differed greatly from these yeasts, whereas similarity was found with codon usage in filamentous fungi.

3.

High-copy-number integration into the ribosomal DNA of the yeast *Phaffia rhodozyma*

(Yeast; astaxanthin; transformation; geneticin; homologous integration; mitotic stability; genomic organization)

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Summary

This report describes a transformation system leading to stable high copy number integration into the ribosomal DNA (rDNA) of the astaxanthin producing yeast *Phaffia rhodozyma*. A plasmid was constructed that contains the transposon Tn5 encoded kanamycin resistance gene (Km^R) fused in frame to the 5' terminal portion of the *Phaffia* actin gene. This marker, driven by the *Phaffia* actin promoter, confers resistance to G418 (Geneticin). The plasmid also contains a rDNA portion that comprises the 18S rDNA and promotes high copy integration leading to stable *Phaffia* transformants, that maintained the plasmid at high copy number after 15 generations of non-selective growth. *Phaffia*, strain CBS 6938, was found to contain the rDNA units in clusters distributed over 3 chromosomes with a total copy number of 61. *Phaffia* transformants were shown to have over 50 copies of pGB-Ph9 integrated in tandem in chromosomes that contain rDNA loci. The chromosomal shifts that occur as a result of these integrations as shown by pulsed field electrophoresis strongly suggest that *Phaffia* is haploid.

Introduction

Phaffia rhodozyma is an orange-red yeast, that produces astaxanthin as the main carotenoid (Miller et al., 1976; Phaff et al., 1972). Based on classical ultrastructural and chemotaxonomic observations (Miller et al., 1976; Sugiyama et al., 1985) and the analysis of the sequence and structure of the actin gene (**Chapter 2**), *Phaffia* is accommodated in the basidiomycetous yeasts.

Astaxanthin has become of great commercial interest to segments of the fish farming industries, since the colorant is responsible for the pink pigmentation of salmon and trout. Furthermore, a possible role of astaxanthin in the prevention of human cancers and other degenerative diseases was demonstrated by Jyonouchi et al. (1995). The increasing economic importance of astaxanthin and the fact that *Phaffia* is one of very few organisms known to synthesize astaxanthin, has evoked the interest of industries in this yeast as a possible source of profitable astaxanthin production, as an alternative for the tedious and costly production of chemically synthesized astaxanthin (Englert et al, 1977; Bernhard, 1991). A drawback however, is the low astaxanthin production (400 µg/g dry weight) in wild type *Phaffia* strains (Johnson and Lewis, 1979), whilst the use of astaxanthin overproducing *Phaffia* mutants is impeded by their genetic instability and lower cell yield (An et al., 1989).

A transformation system for *Phaffia* leading to high-copy-number integration could be of great value to establish stable overproduction of astaxanthin. Recently a successful

transformation of *Phaffia* was described (Adrio and Veiga, 1995) using a plasmid without homologous sequences. Transformants were obtained with low efficiency, maintaining the plasmid episomally with low stability.

The aim of this study was to obtain a transformation system for *Phaffia* leading to high-copy-number integration and stable transformants. Therefore, a plasmid was constructed, that holds a 3-kb *Phaffia* rDNA portion and the amino glycoside phosphotransferase gene (Km^R) driven by the *Phaffia* actin promoter. Transformants were analyzed to determine the mode of plasmid maintenance, sites of integration, plasmid copy number and stability.

Results and Discussion

Development af a transformation system for *Phaffia*

Cloning of a selectable marker: an actin-Km^R fusion driven by the actin promoter

Due to a lack of well defined auxotrophic *Phaffia* strains we determined the sensitivity for various antibiotics. We found that *Phaffia* was sensitive for G418 (Geneticin) at low concentrations. Therefore we decided to use the Km^R gene as a selectable marker in a first step towards the development of a transformable plasmid. This transposon Tn5 encoded gene confers kanamycin resistance to *Escherchia coli* (Jorgensen et al., 1979) and G418 resistance to *Saccharomyces cerevisiae* (Jimenez and Davies, 1980) and a variety of other eukaryotes including mammalian cells (Southern and Berg, 1982), provided a suitable promoter is present.

To ensure expression of the Km^R gene we used the homologous *Phaffia* actin promoter. The full length *Phaffia* actin gene (designated as *act*1) and promoter (*act*1P) have been previously cloned divided over two plasmids pGB-Ph1 and pGB-Ph2 (Chapter 2)(Fig. 1). From these plasmids a construct pGB-Ph7 was obtained in which the Km^R gene was fused in frame to a 0.9-kb 5' portion of *act*1, coding for 83 amino acids of the N-terminus of the *Phaffia* actin. It has been described previously that Km^R fusions work well (Chen and Fukuhara, 1988). The act1/Km^R gene in pGB-Ph7 is driven by the *Phaffia* actin promoter (Fig. 1).

Fig. 1.
Cloning scheme of pGB-Ph9. PTZ18R (Pharmacia) was used as a starting plasmid for all cloning steps. Insert sequences originating from fractionated total *Phaffia* DNA were isolated from 0.7% agarose gels by agarase treatment according to the supplier (Boehringer). DNA digestions and ligations were performed using enzymes purchased from BRL and applied according to the suppliers recommendations. Transformation of *E. coli* JM 109 was done according to the DMSO procedure by Chung et al. (1989). Positive colonies were generally picked up by colony lifting and hybridization using ^{32}P-labeled insert specific probes according to the Colony/Plaque Screen™ manual by Du Pont. Plasmid DNA was isolated according to standard methods described by Sambrook et al. (1989).
The actin gene and promoter of *Phaffia* were previously cloned in plasmid pGB-Ph1 and pGB-Ph2 respectively (Chapter 2). After deletion of a *Xho*I internal fragment from pGB-Ph1 plasmid pGB-Ph3 was obtained. A 1.1-kb *Bam*HI fragment containing the Km^R gene was cloned in the *Bgl*II site, yielding plasmid pGB-Ph5, that contains the Km^R gene downstream and in frame with the 0.9-kb 5' portion of the *Phaffia* actin gene (*act*1). The actin promoter (*act*1P) was placed in front of this fusion by cloning a 1.8-kb *Hind*III-*Sal*I from pGB-Ph2, containing *act*1P, in the *Sal*I-*Hind*III digested pGB-Ph5, yielding plasmid pGB-Ph7. A 3-kb *Sac*I *Phaffia* rDNA fragment was isolated after hybridization with a 1.7-kb *Cla*I rDNA fragment from *K. lactis* (result not shown) and cloned into the *Sst*I site of pGB-Ph7 yielding pGB-Ph9.

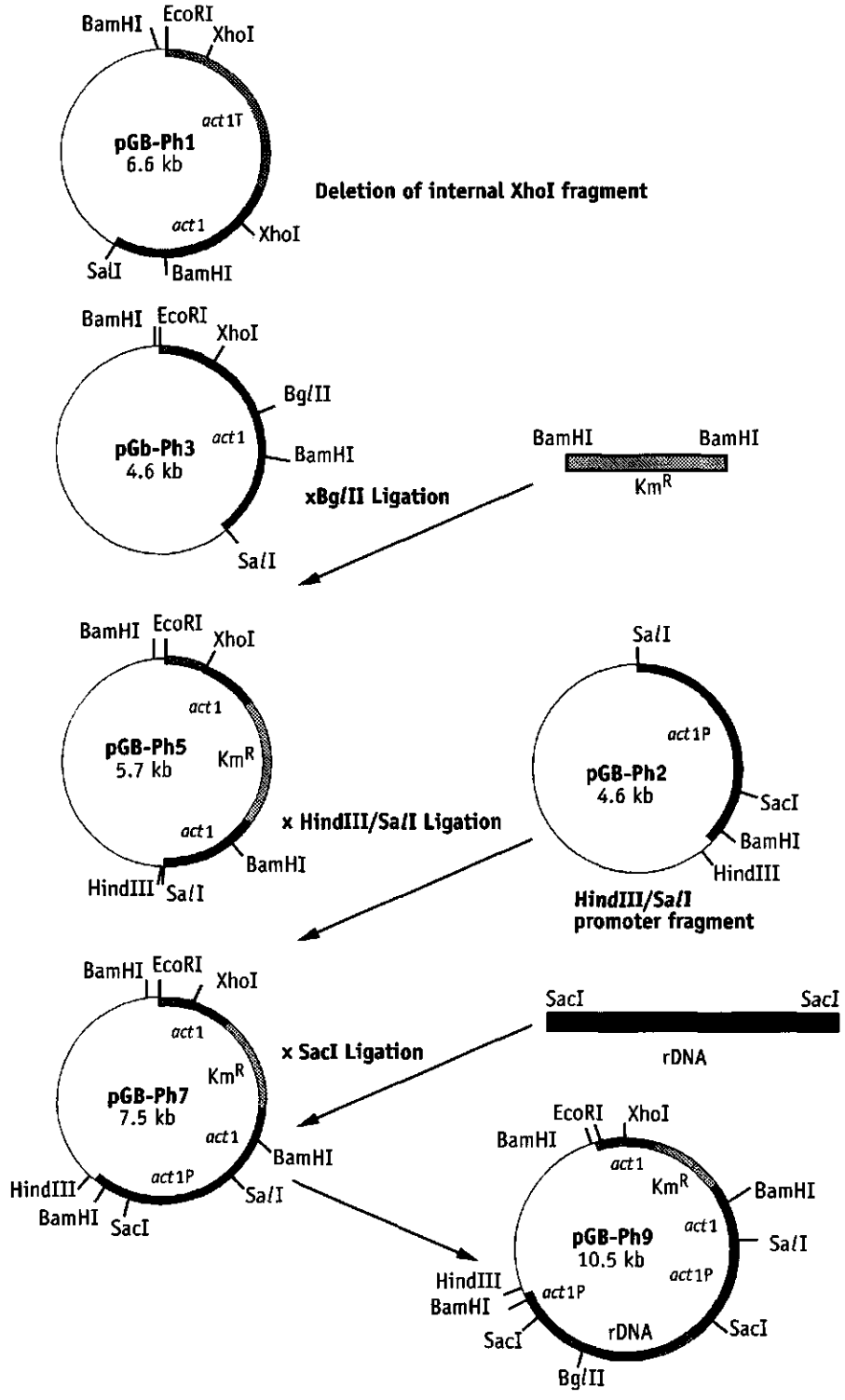
BamHI
EcoRI
XhoI
pGB-Ph1
6.6 kb
act1T
act1
XhoI
SalI
BamHI
Deletion of internal XhoI fragment
BamHI
EcoRI
XhoI
BglII
pGb-Ph3
4.6 kb
act1
BamHI
SalI
xBglII Ligation
BamHI
BamHI
KmR
BamHI
EcoRI
XhoI
act1
pGB-Ph5
5.7 kb
KmR
act1
BamHI
HindIII
SalI
x HindIII/SalI Ligation
SalI
act1P
pGB-Ph2
4.6 kb
SacI
BamHI
HindIII
HindIII/SalI
promoter fragment
BamHI
EcoRI
XhoI
act1
pGB-Ph7
7.5 kb
KmR
act1
BamHI
act1P
HindIII
BamHI
SacI
SalI
x SacI Ligation
SacI
SacI
rDNA
EcoRI
XhoI
BamHI
act1
KmR
BamHI
pGB-Ph9
10.5 kb
act1
SalI
act1P
HindIII
BamHI
act1P
rDNA
SacI
SacI
BglII

FIG. 1.

Cloning of a *Phaffia* rDNA portion to promote high-copy-number integration and stability

Plasmid pGB-Ph7 carries the *act1*/Km^R fusion marker, driven by *act1*P. As we were interested in stable high-copy-number integration, it was decided to isolate *Phaffia* rDNA.

It has been reported previously that in the yeasts *S. cerevisiae*, *Kluyveromyces lactis*, *Yarrowina lipolytica* and *Candida utilis* plasmids containing rDNA sequences are targeted to the rDNA locus of the host cell and integrate, giving rise to stable transformants (Szostak and Wu, 1979; Lopes et al., 1989; Bergkamp et al., 1992; Rossolini et al., 1992; Le Dall et al., 1994; Kondo et al., 1995). It is also known, that in *S. cerevisiae* the rDNA locus contains an autonomous replication sequence (ARS) (Szostak and Wu, 1979). Thus cloning of a *Phaffia* rDNA fragment in pGB-Ph7 could promote either episomal replication or integration in the rDNA of *Phaffia*. A 3-kb *Phaffia* chromosomal *Sac*I DNA fragment, hybridizing with *K. lactis* rDNA (not shown) was cloned in the *Sac*I site of pGB-Ph7, yielding pGB-Ph9 (**Fig. 1**). The rDNA fragment was sequenced and found to contain the entire 18S rDNA, based on comparison with several 18S rDNA's from other yeasts and fungi (result not shown).

Transformation of *Phaffia* with pGB-Ph9

The sensitivity of *Phaffia*, strain CBS 6938, for G418 was determined. We found that growth inhibition of *Phaffia* cells on G418 containing agar plates depended on the cell density.

Using 10^7 cells per plate, anticipating the amount of cells that are plated in our transformation experiments, no background growth was observed at 40 µg/ml G418. Transformation of *Phaffia* with pGB-Ph9 could be successfully performed according to both the method of Elble et al. (1992), with modifications (**Fig. 2**) and the electroporation method as described by Faber et al. (1994).

Both circular and *Bgl*II linearized pGB-Ph9 were transformed. Since pGB-Ph9 contains a unique *Bgl*II site in the rDNA portion, located in the 18S rDNA gene, the use of *Bgl*II linearized plasmid could facilitate integration in the rDNA locus of the host. Indeed transformants were obtained at a frequency of 5/µg transforming DNA using linearized plasmid. No transformants could be obtained using circular plasmid.

Analysis of transformants; pGB-Ph9 is stably integrated at high-copy-number in the genomic DNA of *Phaffia* transformants

To examine the presence of plasmid pGB-Ph9, 15 transformant colonies were cultivated under selective pressure and chromosomal DNA was isolated. After digestion with *Bam*HI and gel electrophoresis, the DNA was transferred to nitro-cellulose and hybridized with a *Phaffia* rDNA probe (**Fig. 2**). From 15 DNA isolates 12 were shown to contain a pGB-Ph9 specific 5.2-kb *Bam*HI DNA fragment containing the 3-kb rDNA sequence.

The copy number of pGB-Ph9 in the transformants varied as deduced from the hybridization signal intensities. It can be concluded, by comparing the hybridization signals of the chromosomal rDNA and the plasmid specific rDNA, that all transformants carried multiple copies of the plasmid.

Phaffia transformants were only obtained using linearized plasmid, indicating an integration event and suggesting the absence of an ARS. Furthermore *Phaffia* transformants maintained their resistance to G418 after growing for over 15 generations under

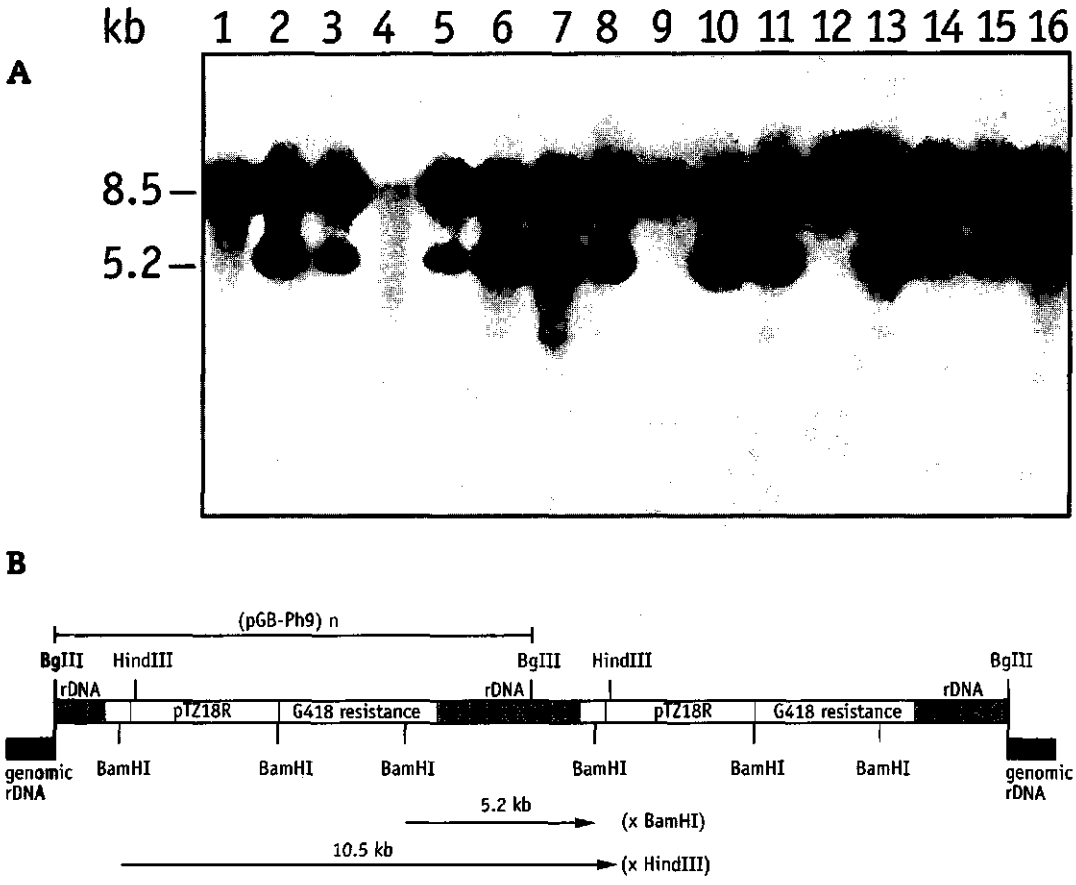

FIG. 2.

Presence of plasmid in several *Phaffia* transformants. Transformation of *Phaffia rhodozyma* strain CBS 6938 was performed using a procedure as previously described (Elble, 1992) with following modifications: 10^7 early logphase *Phaffia* cells and 10 µg transforming plasmid DNA were used per transformation. Incubation of the cell/DNA mixture in PLATE (poly ethylene glycol/lithium acetate/Tris-EDTA) took place during 40 hours at 21°C. After this incubation the cells were harvested and resuspended in 0.5 ml YPD medium. Following a 2-h incubation at 21°C, the cells were spread on selective YPD agar plates containing 40 µg/ml G418. Total DNA from 15 *Phaffia* transformants was digested with *Bam*HI. Following agarose gel electrophoresis, the digested DNA was transferred to nitrocellulose filter and hybridized with a ^{32}P-labeled *Phaffia* rDNA probe, mainly according to Sambrook et al. (1989). The autoradiogram was scanned with a Magiscan 2 apparatus and the intensity of the bands was quantified using the computer programme Genias (General Image Analysis Software). **(A)** Lane 1 contains *Bam*HI digested total DNA from *Phaffia*, strain CBS 6938; Lanes **2-16** contain *Bam*HI digested total DNA from independent *Phaffia* pGB-Ph9 transformants. The number of plasmid copies as deduced from the comparison of the hybridization signals are depicted for each transformant, assuming 61 copies of rDNA within the parental strain. Lane **2**, 43; **3**, 20; **4**, no DNA; **5**, 14; **6**, 45; **7**, 45; **8**, 26; **9**, 0; **10**, 52; **11**, 34; **12**, 0; **13**, 46; **14**, 29; **15**, 27; **16**, 46. A schematic map is drawn in **(B)**, illustrating that multiple copies of plasmid are tandemly integrated in the genomic rDNA of *Phaffia* transformants. The linearizing enzyme *Bgl*II and the enzymes used for hybridization studies (*Bam*HI and *Hind*III) are depicted. Legend: G418 resistance, *act*1/Km^R fusion, driven by the actin promoter; n, number of copies.

non-selective conditions (results not shown). This is in agreement with earlier findings, that integration leads to stable transformants, maintaining their genotype even after many generations of non selective growth (Bergkamp et al., 1992; Lopes et al., 1989).

To further examine the mode of maintenance of the plasmid, DNA isolated from two *Phaffia* transformants, G418-1 and G418-2, grown under selective and non-selective conditions (15 generations), was analysed by gel electrophoresis. Results obtained with G418-1 DNA are depicted in FIGURE 3.

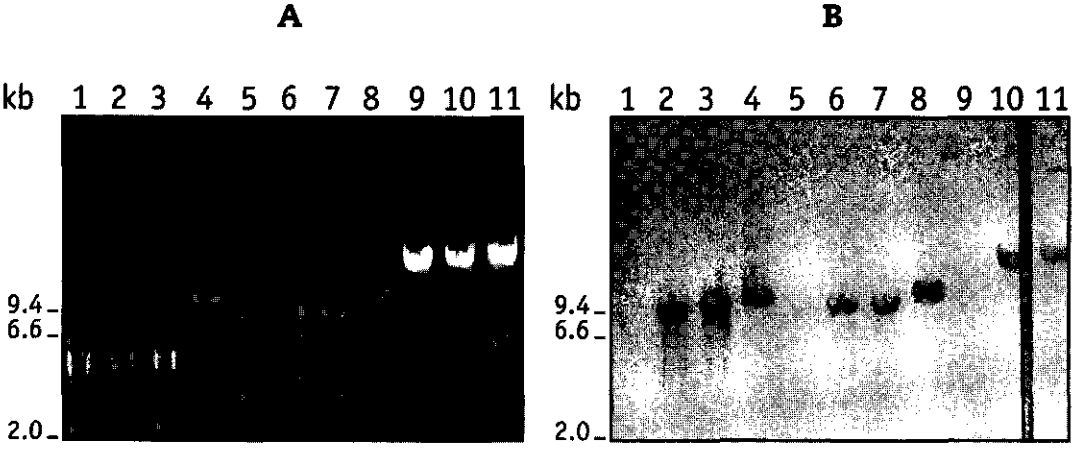

FIG. 3.
Maintainance of plasmid copy number in a *Phaffia* transformant after long term non-selective growth. *Phaffia rhodozyma* strain CBS 6938 was cultivated in YPD medium. Total DNA was isolated, and digested with *Bgl*II and *Hin*dIII. The DNA's were analyzed by agarose gel electrophoresis followed by ethidium bromide staining **(A)**. *Phaffia* transformant G418-1 was grown in either selective YPD medium, containing 50 µg/ml G418, and non-selective YPD medium, for 15 generations. Total DNA from cells from both cultures was isolated, digested with *Bgl*II and *Hind*III and analyzed **(A)**. The DNA's were subsequently transferred to Nytran NY-13-N filters (Schleicher and Schuell) and hybridized with pTZ18R using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim Biochemica) following the suppliers recommendations **(B)**.
(A) Ethidium bromide stained agarose gel. Lanes **1-3** contain *Bgl*II digested total DNA from: **1**, wild type *Phaffia*; **2**, transformant G418-1 (selective); **3**, G418-1 (non-selective). Lanes **5-7** contain *Hin*dIII digested total DNA from **5**, wildtype *Phaffia*; **6**, transformant G418-1 (selective); **7**, G418-1 (non-selective). Lanes **9-11** contain undigested total DNA from **9**, wildtype *Phaffia*; **10**, transformant G418-1 (selective); **11**, G418-1 (non-selective). lanes **4** and **8** contain *Bgl*II digested pGB-Ph9.
(B) Blot of agarose gel as shown in **(A)**.

In the lanes containing total undigested DNA only the high molecular weight DNA hybridizes with a plasmid specific probe. There is no hybridization signal present in the lower plasmid specific regions, which shows that pGB-Ph9 is mostly integrated.

In the lanes with *Bgl*II digested chromosomal DNA from transformant G418-1 a 10.5-kb fragment was prominently visible, which was identified as pGB-Ph9 by hybridization (FIG. 3). This result shows that the *Bgl*II linearization site has been correctly repaired on

the chromosomal rDNA copy by gap repair. Digestion of G418-1 DNA with *Hind*III also yielded a band of 10.5-kb. Since pGB-PH9 contains a single *Hind*III site, this indicates that the plasmid is integrated in tandem (FIG. 2B).

The intensities of the bands representing pGB-Ph9 and the host rDNA in the ethidium bromide stained gel are similar, revealing high copy number integration (FIG. 3). An identical pattern is observed in the case of DNA isolated from G418-1 cells after long term non-selective growth. Since the intensity of the plasmid band is not affected, it can be concluded that the plasmid copy number is maintained. Similar results were obtained with transformant G418-2.

It has been shown previously (Lopes et al., 1989) that high copy number integration of plasmid into the rDNA coincides with low expression of the marker gene. To investigate this, the resistance of *Phaffia* transformant G418-1 was determined by exposure to increasing concentrations of G418 (TABLE 1).

At a concentration of 600 µg/ml G418 none of the plated cells survived. It has been reported previously that *S. cerevisiae* and *K. lactis* transformants, carrying the Km^R gene driven by its natural promoter on episomally maintained plasmids, are resistant G418-concentrations up to 1 mg/ml (Chen and Fukuhara, 1988; Das and Hollenberg, 1982; Jimenez and Davies, 1980). Thus it can be concluded, that despite high copy integration of pGB-Ph9, G418-1 shows a rather limited resistance to G418.

This also could explain the low efficiency of transformation. Apparently transformation of *Phaffia* will only be successful if the copy number of pGB-Ph9 has reached a critical point in early stages. Any cell that carries less copies will be killed off by G418.

TABLE 1. G418 resistance of *Phaffia* transformant G418-1[a]

[G418] µg/ml	*Phaffia* G418-1		Phaffia (CBS6938)
	Dil. = 10^{-4} (OD_{600}=7)	Dil.=10^{-5} (OD_{600}=7)	Dil.=0 (OD_{600}=5)
0	>300	74	>300
200	>300	61	0
300	>300	13	
400	212	2	
500	10	0	
600	0		

[a] 100 µl of two dilutions of exponentially growing *Phaffia* cells were plated on agar medium slants containing different amounts of G418. Culture conditions used; wild type *Phaffia* was cultivated at 21°C in YPD medium containing 1% yeast extract, 2% bacto peptone, 2% glucose. *Phaffia* transformant G418-1 was cultivated in YPD containing 50 µg/ml G418. Plates containing G418 (YPD + 2% agar) were obtained by adding a filter sterile 1000x concentrated solution of G418 in water to hand warm YPD agar medium before plating. The plated *Phaffia* cells were allowed to grow for 3 days at 21°C.

Plasmid pGB-Ph9 integrates in rDNA clusters, that are distributed over 3 chromosomes in the *Phaffia* genome

The genomic organization of the multiple rDNA genes in *Phaffia*, including location and copy number, was unknown.

In the ascomycetous yeasts studied so far, like *S. cerevisiae* (Petes, 1979), *K. lactis*, *K. wickerhamii*, *Pachysolen tannophilus*, *Schizosaccharomyces pombe* and *Torulaspora delbrückii* (Maleszka and Clark-Walker, 1993) as well as the ascomycetous fungi *Aspergillus niger*, *Aspergillus nidulans* (Debets et al., 1990), and *Podospora anserina* (Osiewacz et al., 1990) the rDNA is organized in clusters that are mainly located on a single chromosome. However, the zygomycete *Absidia glauca* (Kayser and Wöstemeyer, 1991), the basidiomycete *Agaricus bisporus* (Lodder et al., 1993) and the basidiomycetous yeast *Candida Glabrata* (Maleszka and Clark-Walker, 1993) are examples of the fewer fungi known to carry rDNA clusters on different chromosomes (TABLE 2).

The location of the rDNA genes in *Phaffia* was determined by separating the chromosomes by means of contour-clamped homogeneous electric field electrophoresis (CHEF) followed by hybidization with the 3-kb SacI *Phaffia* rDNA fragment from pGB-Ph9 (**Fig. 4**).

TABLE 2. Location and copy number of rDNA units in different yeats and fungi

Organism	Y/F	A/B	rDNA Copy nr.	Chrom.	Ref.
S. cerevisiae	Y	A	140	1	1,2
K. lactis	Y	A	68	1	3
K. wickerhamii	Y	A	72	1	3
P. tannophilus	Y	A	28	1	3
T. delbrückii	Y	A	115	1	3
S. pombe	Y	A	117	1	3
Y. lipolytica	Y	A	-	1	4
P. anserina	F	A	-	1	5,6
A. nidulans	F	A	60	1	7,8
A. niger	F	A	-	1	8
A. glauca	F	Z	-	4	9
A. bisporus	F	B	-	2	10,11
C. glabrata	Y	B	100	2	3
P. rhodozyma	Y	B	61	3	This report

Abbreviations: A, ascomycete/ascomycetous; B, basidiomycete/basidiomycetous; Chrom., numbers of chromosomes carrying rDNA repeats; F, filamentous fungus; Ref., references; Y, yeast; Z, zygomycete. References cited: 1. Rubin and Sulston, 1973; 2. Petes, 1979; 3. Maleska and Clark-Walker, 1993; 4. Fournier et al., 1986; 5. Osiewacz et al., 1990; 6. Javerzat et al., 1993; 7. Timberlake, 1978; 8. Debets et al., 1990; 9. Kayser and Wöstemeyer, 1991; 10. Royer et al., 1992; 11. Lodder et al. 1993

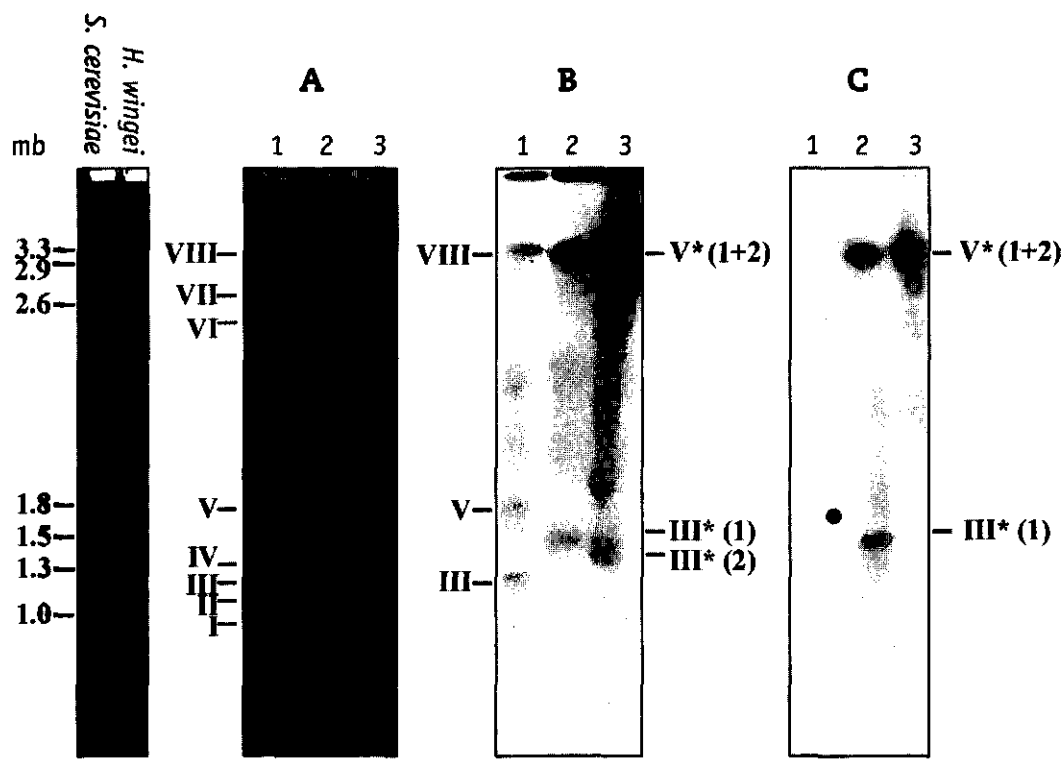

FIG. 4.

Localization of rDNA clusters in the *Phaffia* genome and sites of integration of plasmid pGB-Ph9 in *Phaffia* transformants G418-1 and G418-2. Chromosomes from wild type *Phaffia*, strain CBS6938, and transformants G418-1 and G418-2 were separated by means of countour-clamped homogeneous electric field (CHEF) electrophoresis **(A)**, hybridized with an rDNA probe **(B)** and a Km^R gene specific probe **(C)**. Preparation of chromosomal samples and pulsed field gel electrophoresis, using the Gene Navigator™ system (Pharmacia-LKB), were carried out as described previously (Nagy et al., 1994). Chromosomal DNA from *S. cerevisiae* and *Hansenula wingei* (purchased from Gibco-BRL) were used as size markers. DNA bands representing *Phaffia* chromosomes are denoted by Roman numerals. Asteriks indicate shifts of chromosomal bands from transformants G418-1 (1) and G418-2 (2). Lane **1**, chromosomal DNA from *Phaffia* strain CBS 6938; lane **2**, chromosomal DNA from *Phaffia* transformant G418-1; lane **3**, chromosomal DNA from G418-2.

The CHEF gel showed 8 chromosomal bands, numbered from I to VIII, that range in length from 1 to 3.4 Mb, which is in accordance with the results of Nagy et al. (1994). The band between the chromosomes labeled V and VI is considered to be a result of degradation rather than of a separate chromosome as its presence could not be confirmed in other CHEF analyses.

After hybridization with the rDNA probe (**FIG. 4, PANEL B**) 3 chromosomes (III, V and VIII), sizing 1.2, 1.7 and 3.4 Mb, gave a signal, indicating that the rDNA clusters in *Phaffia* are distributed over 3 chromosomes. This result conflicts with the previous finding that the rDNA units were to be located mainly on 1 chromosome (Nagy et al., 1994). This discrepancy could be explained by the fact that Nagy et al. used a heterologous *Aspergillus*

nidulans rDNA sequence as a probe in their hybridization experiments. Our results support the trend that the rDNA clusters in basidiomycetous yeasts, like *Phaffia*, and fungi studied so far reside on more than one chromosome (TABLE 2).

Besides the location of the rDNA clusters, the copy number of the rDNA units in *Phaffia* was also determined. Great differences exist in rDNA copy number among yeasts. It was shown by Maleszka and Clark-Walker (1993) that among 6 different yeast species rDNA copy numbers varied from 28 (*P. Tannophilus*) to 117 (*S. pombe*), whilst the yeasts *S. cerevisiae* and *K. lactis* contain on the average 150 and 60 copies respectively (Maleszka and Clark-Walker, 1989; Warner, 1989).

The copy number of the rDNA units and the size of the rDNA clusters on the different chromosomes of wild type *Phaffia* was estimated by means of CHEF analysis (FIG. 5). Total DNA of *Phaffia* was digested with *Bst*EII. Since there is no *Bst*EII site in the *Phaffia* rDNA gene (not shown) the rDNA clusters will be excised from the genomic DNA upon digestion with this enzyme. The clusters were separated by CHEF and the gel was stained in ethidium bromide. The gel showed 3 high molecular DNA bands sizing 0.10, 0.12 and 0.30 Mb (FIG 5A, lane 2). They were identified as rDNA stretches after hybridizing with the *Phaffia* rDNA probe (FIG. 5B). The rDNA cluster of *S. cerevisiae*, that is located on the largest chromosome also hybridizes weakly to this probe.

The size of single *Phaffia* rDNA unit is 8.5-kb (FIG. 3), therefore the rDNA clusters account for approximately 12, 14 and 35 copies adding up to a total of 61. In this calculation we have neglected the size of the sequences flanking the rDNA clusters, since *Bst*EII recognizes 6 nucleotides and cuts relatively frequently.

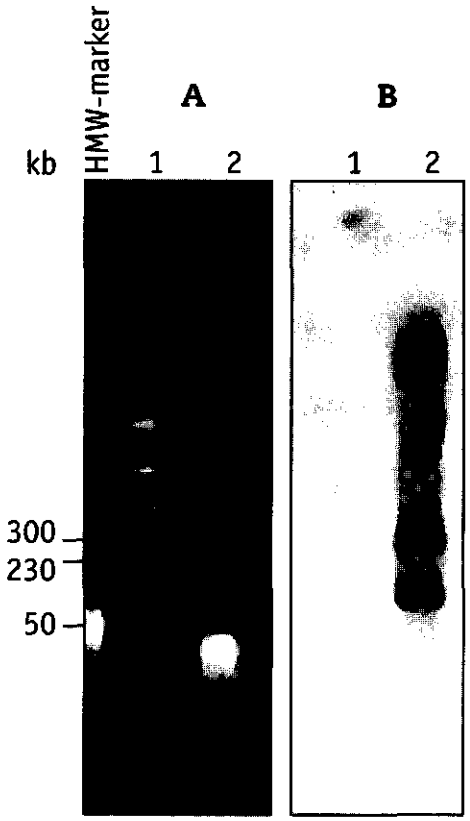

FIG. 5.
CHEF gel analysis of the rDNA clusters of *Phaffia*. Chromosomal DNA from wildtype *Phaffia*, strain CBS6938, was digested with *Bst*EII and separated by CHEF **(A)**.
The CHEF gel was hybridized with a *Phaffia* rDNA probe **(B)**. Separation conditions as described previously **(FIG. 4)** with following adjustments: switching times and voltage used: 200 V for 20 h with a switching time of 90 s. Lane **1** contains chromosomes of *S. cerevisiae*; lane **2** contains chromosomal DNA digested with *Bst*EII from wildtype *Phaffia*.

In order to investigate whether plasmid pGB-Ph9 is targeted to the rDNA loci during transformation, the chromosomes of transformants G418-1 and G418-2 were analyzed by CHEF (FIG. 4). Since it was already established that these transformants carried pGB-Ph9 at high copy number, changes in the electrophoretic migration of especially the chromosomes carrying the rDNA repeats were anticipated.

Indeed, both transformants differ from wild type in that the chromosomes III and V, containing rDNA loci, are shifted to higher molecular weights (FIG. 4, panel A). For transformant G418-1 the size of chromosome III has increased by 0.2 Mb to 1.4 Mb, whereas the size of chromosome V has increased by 1.2 Mb to 2.9 Mb. This was confirmed by hybridization with an rDNA probe (FIG. 4, panel B). For transformant G418-2, hybridization signals are present at approximately 1.3 mb and 2.9 mb after hybridizing with the rDNA probe (FIG. 4, panel A and B).

It was shown previously for *K. lactis* (Maleszka and Clark-Walker, 1990) and *N. crassa* (Russell and Rodland, 1986), that the rDNA copynumber may vary among different strains of the same species, which could affect the electrophoretic migration of chromosomes carrying rDNA. Therefore hybridization with a Km^R gene specific probe was performed additionally to confirm the integration of plasmid in these chromosomes (FIG. 4, panel C). In case of transformant G418-1, both shifted chromosomal bands hybridized with this probe. However, in case of transformant G418-2, a plasmid specific signal is solely present at approximately 2.9 mb. At the position of the other shifted chromosomal band (1.3 mb), that hybridized with with the rDNA probe, no signal is observed. This result suggests that this chromosomal band shift is rather a result of a variation in the size of the rDNA cluster than an integration event.

In conclusion, *Phaffia* transformants were shown to have multiple copies of plasmid integrated in chromosomes carrying rDNA clusters. One transformant, G418-1, was shown to carry multiple plasmid copies in at least two chromosomes, whereas in transformant G418-2 multiple copies of plasmid were integrated in at least one chromosome. As a result the molecular weight of these chromosomes increased. The integration of few plasmid copies in the large rDNA carrying chromosome VIII could not be ruled out. In addition, it was shown that the change of the chromosomal molecular weight may not be solely due to the integration of plasmid. Other effects like rDNA cluster variation may also play a role. Therefore, the increase of the molecular weight of the chromosomes that were shown to contain plasmid DNA may not be proportional to the number of plasmid copies integrated.

Phaffia, CBS 6938, shows haploid features

Till date the ploidy of *Phaffia* was unknown. Several reports show conflicting evidence in this matter. It was suggested by Chun et al. (1992) that based on DNA content, *Phaffia* is diploid. Furthermore, Adrio et al. (1993) reported that auxotrophic *Phaffia* mutants could successfully be obtained only if the potent mutagen N-methyl-N'-nitro-N-nitrosoguanidine was used followed by enrichments techniques. This result also suggested a ploidy higher than haploid. In contrast, Girard et al. (1994), readily obtained great numbers of colour mutants of *Phaffia*, accumulating various carotenoids, by ultraviolet and ethylmethane sulphonate treatment. This result is in accordance with haploidy.

Our results strongly suggest that *Phaffia* CBS 6938 is haploid. The CHEF gel (FIG. 4) clearly shows that chromosome III and V from wild type *Phaffia* can be shifted as a result of high-copy-number integration to higher regions, leaving no chromosome on the original

location. This event is most likely to occur if the chromosomes carrying the rDNA clusters are uniquely present and thus be part of a haploid genome.

CONCLUSIONS

An integrative transformation system for *Phaffia rhodozyma* has been developed, using a vector that confers G418 resistance to *Phaffia* and contains rDNA sequences promoting stable, high-copy-number integration. These features make this transformation system very useful for the overexpression of genes involved in e.g. rate limiting conversions in the astaxanthin biosynthesis pathway of *Phaffia*.

In addition, to our knowledge *Phaffia* is the only basidiomycetous yeast for which a transformation system has been developed, which leads to high-copy-number integration. Since *Phaffia* differs, both physiologically and genetically, from ascomycetous yeasts that were traditionally used as a host for recombinant protein production, like *S. cerevisiae*, *Sz. pombe* and *K. lactis*, this may offer new possibilities for the overexpression of either new genes or genes that are poorly expressed in the organisms used sofar.

The finding that the multiple rDNA units in *Phaffia* are clustered in three repeats divided over 3 different chromosomes adds to the many differences with the common yeasts. This has affected the strategy for development of a transformation system for *Phaffia*.

Although a short description of such transformation system was given in this Chapter, a rationale for the strategy used and the manner in which the increasing genetic knowledge of *Phaffia* has contributed to this system is described in the next CHAPTER 4. In addition, methodologies in yeast transformation and their use for the development of a genetic transformation for *Phaffia* are discussed in detail.

4.

Towards an efficient genetic transformation system for *Phaffia rhodozyma*

Jan Wery, Jan C. Verdoes and Albert J. J. van Ooyen

Introduction

The yeast *Saccharomyces cerevisiae* has been extensively studied on the molecular level and has been accessible for recombinant DNA techniques since its the development as a host for transformations, almost 20 years ago (Beggs, 1978; Hinnen et al., 1978).
The vast genetic knowledge about *S. cerevisiae* and the many genes isolated have been useful in the development of transformation systems for an increasing number of industrially important yeasts, that all share ascomycetous features with *S. cerevisiae*.
In Chapter 3 integration of plasmid in the *Phaffia* genome was already established, however with low efficiency.
In this Chapter the strategies towards the development of an efficient transformation system for *Phaffia* is described. Specific methods and problems in the different stages will be discussed and a comparison is made with the development of basic transformation systems for the industrially important yeasts *Kluyveromyces lactis*, *Pichia pastoris*, *Hansenula polymorpha* and *Yarrowia lipolytica* to provide insight in the considerations for choosing a specific strategy.

Development of a transformation system (in general)

Introduction of foreign DNA

A transformation system depends on, first, the introduction, second, the maintenance of foreign DNA in the host organism and third, selection for this event.

For the introduction of the foreign plasmid DNA into cells transformation methods are described for other organisms. In general, the introduction of foreign DNA can be achieved upon chemical treatment of the host cells. For *Escherichia coli* treatment with divalent cations like Ca^{2+} ($CaCl_2$) alone or in combination with dimethyl sulphoxide (DMSO), hexaminecobalt, and dithiothreitol, are most commonly used for obtaining cells that are highly competent for taking up foreign DNA (Cohen et al., 1972; Hanahan, 1983). For yeast, sole treatment with these chemicals proved not to be sufficient, moreover, the yeast cell wall appeared to be a major obstacle for the passage of DNA.

It was shown by Hinnen et al. (1978) that enzymatic degradation of the cell wall in a hypertonic sorbitol solution, generating protoplasts, followed by treatment with poly ethylene glycol (PEG) and $CaCl_2$, was a prerequisite for obtaining highly competent cells. Although highly efficient, especially the preparation and regeneration of protoplasts proved to be somewhat tedious and laborious.

More recently a simplified procedure for the preparation of intact competent yeast cells was achieved by treatment with the monovalent cation Li^+ (LiCl and LiAc) in combination with PEG (Ito et al., 1983). This method was optimized using high concentrations of denatured carrier DNA (Schiestl and Gietz, 1989).

The most recently developed method for yeast transformation (Delorme, 1989; Meil-

hoc et al., 1990; Becker and Quarente, 1991) is based on exposure of cells, in the presence of the transforming DNA, to high-voltage electric discharges, creating pores in the cell membrane that allow passage of the DNA. This electroporation had been well established for introducing macromolecules into mammalian cells since 1975 (Rieman et al., 1975) and more recently for *E. coli* (Dower et al., 1988).

Plasmid maintenance

In TABLE 1 examples of basic plasmids for *K. lactis*, *P. pastoris*, *H. polymorpha* and *Y. lipolytica* and their properties are summarized. Several of these plasmids have been further engineered for optimal heterologous gene expression by introducing secretion signals, inducible promoters etc. For a detailed description of the latter following reviews are recommended; for *K. lactis*, (Swinkels et al., 1993); *P. pastoris*, (Romanos, 1995); *H. polymorpha*, (Faber et al., 1995); *Y. lipolytica*, (Heslot, 1990).

For maintenance of plasmid DNA in yeasts two possibilities exist: episomal replication or integration into the chromosomal DNA. Episomally maintained plasmids replicate autonomously and independently from the chromosomal DNA, whereas integrating plasmids replicate along with the chromosomal DNA. In general episomal plasmids transform cells with significantly higher efficiency, for successful transformation is solely dependent on the transfer into the cell. In case of an integrative event at a single locus, transformation efficiencies drop 10^3 to 10^4 fold, as successful transformation is additionally dependent on homologous or heterologous integration.

For yeasts several elements are known to promote episomal replication. Autonomous replication sequences (ARS) have been cloned from *S. cerevisiae* and industrial yeasts like *P. pastoris* (Cregg et al., 1985), *H. polymorpha* (Roggenkamp et al., 1986), *K. lactis* (Das and Hollenberg, 1982) and *Y. lipolytica* (Fournier et al., 1991) (TABLE 1). ARS elements naturally reside in the chromosomal DNA at relatively high frequency (approximately one ARS/50 kb), where they initiate chromosomal replication during the S-phase of the cell. ARS-based vectors, that are present in multiple copies from 1 to 20, are inefficiently transmitted over the next generations under non-selective growth. This instability is a drawback for using these plasmids for industrial applications. In general ARS sequences can be easily isolated by cloning of random genomic DNA fragments and screening for increasing transformation efficiency and instable inheritance (Struhl et al., 1979). In *Y. lipolytica*, however, this procedure was not successful at several instances. In 1991 Fournier et al. isolated two *Y. lipolytica* ARS sequences (ARS 18 and ARS 68) with deviating characteristics, like an exceptionally high stability (TABLE 1).

Another type of episomal maintenance is provided by the 2μ plasmid that naturally occurs in *S. cerevisiae* strains at high copynumbers (100 copies) with high stability. Using 2μ sequences, involved in replication and stabilization, in *E. coli*-yeast shuttle expression vectors, stable high copy number transformants are obtained (Broach, 1983; Parent et al., 1985; Armstrong et al., 1989). For *K. lactis* similar episomal expression vectors have been constructed using a 2μ like plasmid pKD1 (TABLE 1). This system has been successfully used for heterologous gene expression. (Fleer et al., 1993a, Fleer et al., 1993b).

Plasmid maintenance by integration usually involves the targeting of plasmid DNA to a specific locus in the chromosomal DNA. Although the transformation of circular plasmid may yield integration by a double cross-over event, digestion of the plasmid in the homologous sequence prior to transformation, will enhance targeted integration (single cross-over). It is well established in a variety of yeasts that integrated plasmids

are even more stably maintained without selective pressure than 2μ or pKD1 derived plasmids.

In *K. lactis*, systems based on integration in the *LAC4* locus are described for stable heterologous gene expression (Van den Berg et al., 1990). In *P. pastoris* stable heterologous gene expression was obtained by homologous integration into the alcohol oxidase gene (*AOX1*) and the *HIS4* gene (Thill et al., 1990). Furthermore, in *H. polymorpha* plasmids that were initially episomally maintained by either the presence of a homologous or fortituous ARS element were shown to integrate after prolonged cultivation (stabilization). This was accompanied by a transition from rather instable to stable transformants (Roggenkamp et al., 1986). In addition, both heterologous integration and homologous integration in the alcohol oxidase gene (AO) was obtained by transformation of linearized plasmid (Faber et al., 1992). In *Y. lipolytica* linearized plasmids targeted to the *LEU2* gene and the alkaline extra-cellular protease encoding *XPR2* gene are used for efficient homologous integration (Davidow et al., 1985; Gaillardin and Ribet, 1987; Nicaud et al., 1989; Bauer et al., 1993).

In general, integrative transformation yields one to few integrated plasmid copies, which may impose limits on the expression on the foreign gene. Occasionally multicopy integration occurs, especially if high concentrations of transforming plasmid are used.

For *P. pastoris* a procedure was described based on the screening for multiple copy transformants. The transposon 903 encoded kanamycin gene (Km^R) which confers resistance to the antibiotic G418 in a variety of eukaryotes was cloned in a *P. pastoris* expression vector. Since the resistance to G418 is tightly correlated with the copynumber of the Km^R gene, transformants could be screened by exposure to increasing concentrations of G418, carrying 3-18 plasmid copies (**Table 1**).

A different type of integrative transformation is based on the targeting of plasmids to the ribosomal DNA (rDNA). It was found by Szostak and Wu (1979) that rDNA containing plasmids, could transform *S. cerevisiae*, upon linearization within the rDNA portion, with at least 100 fold higher efficiency than linearized plasmids that were targeted to the *LEU* locus (Szostak and Wu, 1979). This was contributed to the fact that yeast rDNA consists of approximately 140 copies, providing a much higher amount of homologous DNA for recombination. Moreover, a part of the rDNA gene cloned promoted high-frequency non-integrative transformation, suggesting the presence of a autonomous replication sequence (ARS). More recently Lopes et al. (1989) and Bergkamp et al. (1992) found for *S. cerevisiae* and *K. lactis* respectively, that rDNA containing plasmids could integrate at high copy numbers (up to 200) with even higher stability than other integrating systems (Bergkamp et al., 1992; Lopes et al., 1989).
After these findings, rDNA has been used as a stabilizing sequence in plasmids for non-*saccharomyces* yeasts like *Candida utilis* (Kondo et al., 1995) and *Y. lipolytica* (Le Dall et al., 1994).

Selection markers

The selection for cells that have acquired the foreign DNA is in many cases based on the presence of a marker gene that complements an auxotrophy of the host (auxotrophic selection marker) or renders resistance to an antibiotic (dominant selection marker).

In the first case a host strain deficient in a particular conversion in the synthesis of e.g. an amino acid (*TRP*, *HIS*, *LEU*, etc.) or a nucleoside (*URA*) will lose this phenotype if complemented by the marker gene coding for this conversion. This event can be easily

TABLE 1. Basic transformation systems for *K. lactis*, *H. polymorpha*, *P. pastoris* and *Y. lipolytica*

Yeast	Plasmid[a]	Marker[b]	Homol./heterol.[c]	Maintenance[d]	Max. traf. eff.[e]	Copy nr.[f]	Stability (%)[g]	Ref.[h]
K. Lactis	pKARS	*TRP1*	het.(Sc)	KARS (ep.)	$3x10^4$	nd	10-15	Das, 1982
	pKS105	Km^R+ADHI prom.	het.(Tn*5*)+het.(Sc)	*LAC4* (hom.int.)	nd	multiple	100 (50 gen.)	Van den Berg, 1990
	YIprD1-LYS	*HIS3*	het.(Sc)	rDNA (repl.int.)	nd	4-40	100	Rossolini, 1992
	pMIRK1	*TRP1*	het.(Sc)	rDNA (add.int.)	nd	60	100	Bergkam, 1992
	pCJX10,11,12	*URA3,TRP1,LEU2*	het.(Sc)	Klori (ep.)	$1.2x10^4$	19	12	Chen, 1996
	pCJX3,5,6	Km^R	het. (Tn903)	Klori (ep.)	$1.1x10^4$	16	18	
	pCJX18,19,20	*URA3,TRP1,LEU2*	het.(Sc)	K1CEN2 (ep.)	$8.2x10^3$	1	37	
H. polymorpha	YEp13	*LEU2*	het.(Sc)	LEU2 ARSact. (ep)	60	nd	8.5-8.6	Gleeson, 1986
	YIp*5*	*URA3*	het.(Sc)	Fortituous ARS/het.int	40	1	100	Roggenkamp, 1990
	YRP17	*URA3*	het.(Sc)	ScARS1 (ep.)	300	5	1	
	pHARS1	*URA3*	het.(Sc)	HARS1 (ep.)	$1.5x10^3$	40	2	
	pHARS2	*URA3*	het.(Sc)	HARS2 (ep.)	460	25 (ep.)	1.5 (ep.)	
				/int.		75 (int.)	100 (int)	
	pHIP1	*LEU2*	het.(Sc)	*LEU2* ARSact. (ep)	$5x10^3$	6	15-45 (40 gen.)	Faber, 1992
	(linear)			het.int.	10^5	1-3	100	
	pHRP2	*LEU2*	het.(Sc)	HARS1 (ep.)	$1.5x10^3$	6	2	
	pHIP11	*LEU2*	het.(Sc)	*LEU2* ARSact. (ep)	$3X10^3$	nd	15-45 (40 gen.)	
	(linear)			hom.int. AO	$4.6x10^4$	1-3	100	
	(linear)			het.int.	$5.6x10^4$	1-3	100	
	pCE36	*LEU2*	het.(Sc)	HARS36 (ep.)	$3x10^3$	nd	3 (ep.)	Sohn, 1996
				/int			100 (int)	
P. pastoris	pYA2	*HIS4*	het.(Sc)	Fortituous ARS	$1.6x10^4$	6	10	Cregg, 1985
	pYA4	*HIS4*	hom.	*LEU2* ARSact. (ep)	$9.7x10^4$	6	29	
	pYJ30	*HIS4*	hom.	PARS1 (ep.)	$1.8x10^5$	13	50	
	pYJ32	*HIS4*	hom.	PARS2 (ep.)	$1.7x10^5$	13	51	
	pPIC3K	Km^R	het. (Tn903)	AOX1 (hom.add.int.)	400	3-8	nd	Scorer, 1993
				AOX1 (hom.repl.int.)	20	7-18	nd	

TABLE 1. *continued*

Yeast	Plasmid[a]	Marker[b]	Homol./heterol.[c]	Maintenance[d]	Max. traf. eff.[e]	Copy nr.[f]	Stability (%)[g]	Ref.[h]
Y. lipolytica	pINA465 (linear)	*LYS2*+ fortituous prom	het.(Sc) hom.	hom./het. int.	10	multiple, tandem	nd	Gaillardin, 1985
	pLD25 (linear)	*LEU2*	hom.	hom. int.	100 10^4	nd	100	Davidow, 1985
	pINA465 (linear)	*LEU2*	hom.	hom. int.	10^4	1	nd	Gaillardin, 1985
	pINA95 (linear)	*LEU2*, Tn*5*ble+*LEU2* prom.	hom. het. (Tn*5*)+hom.	hom. int.	$6.4x10^3$ 80	1	nd	
	pINA98 (linear)	*LEU2*, *lacZ*+*LEU2* prom.	hom.het.(Tn*5*)+hom.	hom. int	100 $5.4x10^4$	1	nd	
	pINA169 (linear)	*SUC2*+*XPR2* prom., *LEU2*	het.(Sc)+ hom. hom.	hom. int.*XPR2*	$2.4x10^4$(suc$^+$) $3.3x10^4$(leu$^+$)	1	nd	Nicaud, 1989
	pINA119	*LEU2*	hom.	ars18	10^4	3	25 (Fil$^-$), 69-96	Fournier, 1991
	pREB53 (linear)	β-GUS+*LEU2* prom., *LEU2*	het.(Ec)+hom.,hom.	hom. int	nd	nd	nd	Bauer, 1993
	pIN 767, 772, 773 (linear)	*URA3*+trunc. prom.	hom	hom. int. rDNA	10-10^3	5-60	80-100	Le Dall, 1994

[a] plasmid name
[b] marker gene relevant for screening
[c] source of the marker gene
[d] mode of plasmid maintenance
[e] transformation efficiency in colonies per μg transforming DNA
[f] number of plasmid copies in transformants
[g] stability in percentage of cells that maintained the plasmid after 10 generations of non-selective cultivation, unless stated otherwise;
[h] source of information
Abbreviations: act. activity; add. additive; AO, *H. polymorpha* alcohol oxidase gene; *AOX1*, *P. pastoris* alcohol oxidase gene; β-GUS, β-Glucuronidase gene; Ec, *E. coli*; ep., episomal; Fil$^-$, in a mutant unable to produce hyphae, het., heterologous; hom., homologous; int., integration; KARS, *K. lactis* ARS; Klori; *K. lactis* replication origin from pKD1; HARS, *H. polymorpha* ARS; repl., replacement; Sc, *S. cerevisiae*; trunc., truncated; *XPR2*, alkaline extracellular protease gene.

selected for using medium lacking the relevant amino acid or nucleoside.

In case no well defined auxotrophic strains are available dominant selectable markers, like the Km^R and ble genes, that confer resistance to antibiotics, can be used if the host for transformation is sensitive.

The Km^R gene, that originates from transposons Tn5 and Tn 903 and codes for a bacterial aminoglycoside 3'phosphotransferase (Berg et al., 1975), is used in *S. cerevisiae*, *K. lactis* and *P. pastoris* (TABLE 1). The selection procedure is based on the resistance to the aminoglycoside antibiotic G418, that is inactivated by this gene.

Y. lipolitica was shown to be insensitive for G418 (Gaillardin and Ribet, 1987). For this yeast the Tn5 encoded ble gene was used as a dominant marker. This gene confers resistance to phleomycin and is used as a marker in many filamentous fungi as well as in *S. cerevisiae* and *Schizosaccharomyces pombe* (Prentice and Kingston, 1992, Wenzel et al., 1992).

Development of a transformation system for *Phaffia*

The opportunistic approach: *using existing heterologous plasmids and transformation procedures*

The main question that arises when developing a transformation system for any "new" organism is what strategy to employ: development of a heterologous system using existing techniques and plasmids or a homologous system, requiring the laborious and time-consuming isolation and sequencing of marker and maintenance sequences.

Since a transformation system usually is only a step in reaching the final goal, for instance isolating genes and production of foreign proteins, it is tempting to choose the fastest option and use plasmids and transformation procedures that already exist. The use of plasmids consisting entirely or partly of heterologous DNA has been shown successful (TABLE 1).

Since most industrial yeasts are more or less related to *S. cerevisiae* in that they share ascomycetous characteristics, the use of *S. cerevisiae* markers, promoters and maintenance sequences has been of help in the development of transformation system for several industrially important yeast species, like *K. lactis*, *H. polymorpha* and *P. pastoris*.

In TABLE 1 is shown that the majority of the auxotrophic markers used in the basic transformation systems for these yeasts in fact originate from *S. cerevisiae*. *P. pastoris HIS4* strains could be efficiently transformed with a plasmid carrying the *S. cerevisiae HIS4* gene, whereas the the *S. cerevisiae TRP1*, *HIS3* and *URA3* genes could be used for efficient transformation of *K. lactis* strains with corresponding auxotrophies. Furthermore, the *S. cerevisiae LEU2* and *URA3* genes have been used to complement leu and ura auxotrophic strains of the methylotrophic yeast *Hansenula polymorpha*.

In few cases heterologous use of plasmid maintenance sequences has also been described. DNA sequences from very diverging eukaryotic species were shown to be capable of promoting high frequency transformation with episomal maintenance in *S. cerevisiae* (Stinchcomb et al., 1980). In addition, the *S. cerevisiae LEU2* gene e.g. has been shown to promote autonomous replication in *P. pastoris* and *H. polymorpha* (Cregg et al., 1985; Berardi and Thomas, 1990; Faber et al., 1992). Fortituous ARS activity may also be present on heterologous plasmid sequences.

Heterologous sequences are also used to drive expression of antibiotic resistance markers. Expression of the Km^R gene was observed in *S. cerevisiae*, *K. lactis* and *P. pastoris* under control of its native bacterial promoter (Das and Hollenberg, 1982, Jiminez and Davies, 1980, Scorer et al., 1993). In addition, Chen and Fukuhara (1988) showed that the

Km^R gene driven by the *S. cerevisiae ADH1* promoter conferred high resistance to G418 in both *S. cerevisiae* and *K. lactis*.

Y. lipolytica appears to be quite different in that most of the auxotrophic markers are homologous. In addition, the expression of the *S. cerevisiae LYS2* and *SUC2*, the bacterial Tn5ble, *lacZ* and β-GUS genes were driven by homologous promoter sequences (TABLE 1).

The examples given here show that, in many instances, DNA encoded properties can be universally applied. Therefore we decided to first try the heterologous approach towards a transformation system for *Phaffia*.

Since no well defined stable auxotrophic *Phaffia* strains were available, it was decided to use the Km^R gene as a dominant selectable marker. First the sensitivity for G418 was determined, since the selection procedure for the transformation was based on the resistance to this antibiotic (TABLE 2).

TABLE 2. Growth inhibition of *Phaffia* by G418

[G418] μg/ml	0	20	30	40	50	100
Colony count [a]	overgrown	overgrown	30	2	0	0

[a]Approximately 10^7 exponentially growing *Phaffia* cells were plated on YPD (1% yeast extract, 2% bacto peptone, 2% glucose) agar slants containing increasing amounts of G418. Colonies were counted after 3 days of incubation at 21°C.

We found that growth inhibition of *Phaffia* cells on G418 containing YPD agar plates was influenced by the cell density. The G418 resistance was determined by plating *Phaffia* cells on YPD agar plates with increasing concentrations of G418. We used 10^7 cells per plate, anticipating the maximum amount of cells to be spread in our transformation experiments. Since background growth was negligible at 40 μg/ml G418, it was decided to select for transformants on YPD plates containing 40 μg/ml G418 in various transformations.

Existing plasmids were used for the transformations, containing the Km^R gene downstream of the native Tn 903 , the *S. cerevisiae ADH1*, or the mammalian simian virus SV 40 promoter. Although no *Phaffia* sequences were present, we anticipated expression in *Phaffia* using these different plasmids, since the gene was previously shown to confer aminoglycoside resistance to a wide variety of prokaryotes and eukaryotes.

The transforming plasmid DNA's were differently prepared. Covalently closed circle forms, purified by CsCl gradient were used anticipating the presence of fortituous replicating sequences. In addition, linearization was performed prior to transformation to enhance integration by single crossing-over.

Since there was no knowledge as to what transformation procedure to use best, we both used the spheroplasting method of Hinnen et al. (1978) and the LiCl method by Ito et al. (1983). However, in both cases no transformants were obtained.

At this stage of research a number of questions arose concerning the cause of the failure to obtain transformants. Are the marker genes on the plasmids used not sufficiently expressed? Do the plasmids lack sequences for maintenance in *Phaffia*? Are the transformation procedures used not suited for *Phaffia*?

It was decided to abandon the heterologous approach, because of the many uncertainties to be solved and, more importantly, because we were committed to work with *Phaffia* on a long term basis.

The secure approach: development of homologous plasmids

Isolation of total *Phaffia* DNA

Before isolating specific *Phaffia* DNA sequences, first a method was developed for the isolation of total DNA. We found that procedures optimized for many ascomycetous yeasts, including *K. lactis*, *P. pastoris*, *H. polymorpha* and *Y. lipolytica*, that were based on protoplasting methods with Zymolyase 20T or 100T, were not suited for *Phaffia*. This was probably caused by the double layered *Phaffia* cell wall and its specific basidiomycetous physico-chemical properties, like the presence of xylose (Sugiyama et al., 1985). A method using Novozym 234 (Novo Industri A/S, Denmark), that was used as a cell wall degrading enzyme for several basidiomycetous yeasts and fungi, was applied for *Phaffia* (CHAPTER 2).

Isolation of the *Phaffia* glyceraldehyde phosphate dehydrogenase (*GPD*) gene

For the development of a homologous transforming plasmid 3 issues raised previously, expression of the marker, plasmid maintenance and the transformation procedure, have to be addressed.

To optimize the chance of expression, the Km^R gene would best be driven be a strong constitutive promoter. For this purpose we decided to isolate a glycolytic gene. Besides a high constitutive expression level, glycolytic genes are well conserved, which is important for the isolation procedure.

For the isolation of these types of genes two strategies can be considered, first heterologous hybridization and second PCR using degenerate primers. The first method usually involves the use of a gene probe from a different species for hybridization with genomic or library DNA. If performed under non-stringent conditions a specific hybridization signal may be obtained. However, if the genes differ too much no hybridization will be obtained. In addition, the risk of picking up aspecific products, as a result of too low stringency hybridization conditions, is real.

This was illustrated by the fact, that in our efforts to pick up the triose phosphate isomerase (*TPI*) and phosphoglycerate kinase (*PGK*) genes by use of heterologous probes from *K. lactis* and *S. cerevisiae*, respectively, we could not obtain a hybridization signal under non-stringent hybridization conditions with chromosomal *Phaffia* DNA. Even the use of the of a *K. lactis* actin probe resulted in only a very weak specific signal (CHAPTER 2), despite the fact that actin belongs to the best conserved genes known.

It was decided to isolate the *Phaffia* glyceraldehyde phosphate dehydrogenase (*GPD*) gene by the alternative approach, a method that involved synthesis of a homologous GPD probe by PCR on total *Phaffia* DNA, followed by screening of a *Phaffia* genomic DNA cosmid library, using this probe (Verdoes et al., 1996).

The key step in this procedure is the choice of the sequence of the oligo nucleotides (primers) used in the PCR. The sequence of the primers must be optimized for specific annealing to ensure optimal amplification of the target DNA. Since the nucleotide sequence of the target DNA (in this case part of the *Phaffia GPD* gene) is unknown, the design of the primers must be derived from conserved regions as determined by comparison of the sequences of several species of this gene from other organisms. In case none or only few species of the concerning gene are sequenced, this method is better not employed, for a reliable estimate of conserved regions cannot be made.

The conserved regions of the *Phaffia GPD* gene, were determined by comparison 11

TABLE 3. Codon usage in *Phaffia* compared with *S. cerevisiae*

Amino acid	codon	In *Phaffia*[a] genes (%)	In S.c.[b] genes (%)	Amino acid	codon	In *Phaffia* genes (%)	In S.c.
Phe	TTT	4	54	Tyr	TAT	0	50
	TTC	96	46		TAC	100	50
Leu	TTA	2	27	His	CAT	2	60
	TTG	15	36		CAC	98	40
	CTT	33	11	Gln	CAA	4	74
	CTC	47	5		CAG	96	26
	CTA	2	13	Asn	AAT	8	55
	CTG	2	9		AAC	92	45
Ile	ATT	20	50	Lys	AAA	4	52
	ATC	80	30		AAG	96	48
	ATA	0	20	Asp	GAT	36	62
Met	ATG	100	100		GAC	64	38
Val	GTT	25	44	Glu	GAA	2	74
	GTC	65	25		GAG	98	26
	GTA	1	16	Cys	TGT	26	67
	GTG	9	15		TGC	74	33
Ser	TCT	25	31	Trp	TGG	100	100
	TCC	58	18	Arg	CGT	1	17
	TCA	2	19		CGC	1	4
	TCG	12	8		CGA	86	5
	AGT	2	15		CGG	2	2
	CGC	1	9		AGA	3	54
Pro	CCT	35	29		AGG	8	17
	CCC	60	13	Gly	GGT	41	60
	CCA	2	49		GGC	7	15
	CCG	3	9		GGA	50	15
Thr	ACT	19	38		GGG	2	9
	ACC	75	24		GCT	35	44
	ACA	2	26		GCC	65	24
	ACG	3	11		GCA	0	24
					GCG	0	8

[a] A total of 10 *Phaffia* genes, including 8 ribosomal protein genes (unpublished), the *GPD* gene (Verdoes et al., 1997) and the actin gene (**CHAPTER 2**), were analyzed.

[b] A total of 484 *S. cerevisiae* (S.c.) genes were analyzed. Data from Zhang et al. (1991)

GPD protein sequences from diverging organisms (Michels et al., 1986). Approximately 10 highly conserved peptide motivs were found throughout the protein. For the design of the PCR primers the DNA sequence of these motivs must be inferred from the amino acid sequence. This procedure is complicated by the fact that the third and sometimes the first nucleotide in a codon may be variable (degenerate codon). This necessitates the use of a mixture of each primer variant in these nucleotides (degenerate primers) in the PCR assay.

The extend to which the primers are degenerate is depended on the differential use of codons by the organism (codon usage). Codon usage is species specific (Andersson and Kurland, 1990, for a review) and can be determined if more genes of a given species have been isolated.

For *Phaffia* the codon usage was determined in the previously isolated actin gene (**CHAPTER 2**) and in 8 ribosomal protein genes (unpublished), that are like the *GPD* gene constitutively expressed. From **TABLE 3** it can be deduced that *Phaffia* has a strong codon preference for the different amino acids, that differs from *S. cerevisiae*.

In general, a C is preferred in the third codon position, whereas an A in this position is strongly under-represented. In case of Gly and Arg the opposite is true. Especially for the 9 amino acids that are represented by 2 codons and for Arg, Thr and Ile, the preference for 1 codon is evident (>75%).

This knowledge significantly facilitates the design of degenerate primers. For Arg, e.g., that is in theory represented by 6 different codons, the use of one (CGA, 86%) would for *Phaffia* be appropriate in a degenerate primer.

The presence of introns, that generally reside at the 5'part of a gene, may interfere with specific priming or may cause amplification of product with an unexpected size. The first will occur if the introns are located within the target sequence of one or both primers, whereas introns residing in the DNA fragment to be amplified, will yield an amplified product that is larger than expected.

Since introns may occur frequently in the *Phaffia* genome, as indicated previously by the presence of 4 introns in the 5'part of the actin gene (**CHAPTER 2**), it was decided to design the upstream and downstream primers based on amino acid motivs occurring at the carboxy terminal end of the *GPD* gene. The motivs chosen, were spaced 90 amino acids, representing approximately 0.3 kb on the gene.

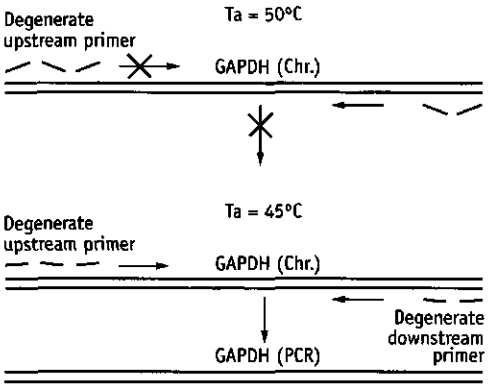

FIG. 1.
Schematic representation of influence of the annealings temperature in a PCR assay using degenerate primers for the amplification of a fragment of the *Phaffia GPD* gene. At 50°C the degenerate primers do not anneal properly to the target sequence on the *GPD* gene, inhibiting first strand synthesis by the Taq polymerase. At 45°C the primers anneal sufficiently for proper amplification. Abbreviations: Ta, annealings temperature; Chr., chromosomal DNA.

A PCR assay with degenerate primers must be optimized for specific product generation. Especially the annealing temperatures used are of importance. Too low temperatures will give rise to aspecific annealing resulting in many nonsense products, whereas no product will be obtained using too high temperatures (**FIG. 1**).

In our trials PCR was performed using annealing temperatures ranging from 40°C-50°C. At 45°C a DNA fragment was generated with an expected size of 0.3 kb, that was confirmed to encode for the carboxy terminal end of the *Phaffia* GPD gene by comparison with the *S. cerevisiae GPD* sequence (Verdoes et al., 1996).

From this critical stage on, the remaining part of the procedure involved straight forward homologous hybridizations using the PCR generated *Phaffia GPD* fragment as a probe for screening a *Phaffia* genomic cosmid library, resulting in the isolation of the complete *Phaffia* GPD gene, including promoter and terminator.

The use of these sequences for the construction of a vector that is efficiently transformed to *Phaffia* is described in **CHAPTER 5**.

5.

Efficient transformation of the astaxanthin producing yeast *Phaffia rhodozyma*

Jan Wery, Jan C. Verdoes and Albert J. J. van Ooyen

Summary

We have developed an efficient transformation system for the astaxanthin producing yeast *Phaffia rhodozyma* based on electroporation, routinely yielding approximately 1000 transformants per µg of plasmid DNA. The high transformation efficiency depended on integration in the ribosomal DNA (rDNA) and the presence of the homologous glycolytic glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter and terminator to drive the expression of the transposon Tn5 encoded kanamycin resistance gene (Km^R) as a selective marker. Using this system stable transformants were obtained, carrying on the average a total of 20 plasmid copies. The plasmid DNA was shown to be integrated in tandem arrays, that were dispersed through the rDNA. Furthermore, the plasmid copy number could be increased to 65 by deletion of the *GPD* terminator from the transforming plasmid. Contrary to earlier findings with other yeasts, like *Saccharomyces cerevisiae*, our experiments suggest that in *Phaffia* high-copy-number (hcn) integration in the rDNA occurs independently from selection pressure.

Introduction

The basidiomycetous yeast *Phaffia rhodozyma* synthesizes the industrially important colorant astaxanthin (Miller et al., 1976, Phaff et al., 1972). Astaxanthin is responsible for the characteristic orange-pink pigmentation of a variety of organisms (fish, birds) through their diet. Chemically synthesized astaxanthin is used by the fish farming industry as a fish feed additive for pen raised salmon and trout. Furthermore, astaxanthin has strong antioxidant properties (Lim et al., 1992, Palozza and Krinsky, 1992) and has been shown to play a role in immune system activation (Jyonouchi et al., 1995) and in the prevention of carcinogenesis and other degenerative diseases (Rousseau et al., 1992; Tanaka et al., 1994; Tanaka et al., 1995; Miller et al., 1996)

Besides algae like *Haematococcus pluvialis* (Boussiba and Vonshak, 1991), and some marine bacteria like *Agrobacterium aurantiacum* (Yokoyama et al., 1994), *Phaffia* is the only micro-organism known to synthesize astaxanthin as the main carotenoid. Since the chemically synthesized astaxanthin is expensive ($2400/kg) and the market for astaxanthin (currently $200 million) is expected to grow significantly, much attention has been given to both physiology of astaxanthin production by *Phaffia* and classical strain improvement in order to provide a microbiological production that can compete with chemical synthesis (An et al., 1989; Fang and Cheng, 1993; Meyer et al., 1993). However, till date the chemical synthesis of astaxanthin is favored mainly due to the low yield of microbial production.

In order to gain more insight in the molecular biology of *Phaffia* we have initiated genetic research on this unconventional yeast, which led to the isolation and characteri-

zation of the conserved actin and GPD genes (CHAPTER 2; Verdoes et al., 1996). Furthermore, the genomic organization of *Phaffia* ribosomal DNA (rDNA) units was elucidated and a plasmid was constructed for *Phaffia* (CHAPTER 3) that could integrate in the rDNA at high-copy-number (hcn), albeit with a very low transformation efficiency.

The use of astaxanthin overproducing *Phaffia* strains, obtained by classical mutagenesis, has its limitations, like genetic instability and low cell yields. Therefore we consider the application of recombinant DNA technology of importance to optimize carotenoid production.

In this report we describe the development of the first transformation system for *Phaffia* that is both efficient and stable and leads to hcn integration. It is based on resistance to the antibiotic Geneticin (G418) and integration into the rDNA. Furthermore, elements in the plasmid used that mediate hcn integration and transformation efficiency were studied. In addition the mode of hcn integration was investigated.

MATERIALS AND METHODS

Strains and media

Escherichia coli strain DH5α was used for transformation and amplification of recombinant plasmids according to standard methods (Sambrook et al., 1989).

Phaffia rhodozyma strain CBS 6938 was cultivated at 21°C in YPD medium containing 1% yeast extract, 2% bactopeptone, 2% glucose. Transformant *Phaffia* strains were cultivated in YPD supplemented with 20 μg/ml Geneticin® (G418 Sulphate, Gibco-BRL). For solid medium 2% bacto-agar was added supplemented with Geneticin® if appropriate.

DNA methods

Insert sequences were isolated from 0.7% agarose gels using the Geneclean® II Kit (BIO 101 Inc.). DNA fragments obtained by PCR were purified from low melting point agarose using the Magic PCR Prep kit (Promega). DNA digestions and ligations were performed using enzymes purchased from BRL and applied according to the suppliers recommendations. For DNA hybridizations total *Phaffia* DNA was digested and transferred to nylon filters according to standard protocols (Sambrook et al., 1989). Hybridizations were performed using the DIG DNA Labeling and Detection Kit, non radioactive (Boehringer). For hybridization signal intensity comparisons autoradiograms were scanned with a Magiscan 2 apparatus and the intensity of the bands was quantified using the computer program Genias (General Image Analysis Software).

Plasmid construction

Plasmid pUCG418 (Van den Berg et al., 1989), was digested with *Kpn*I and *Msc*I. A 596 bp insert, containing the 385 bp 3'part of the *Phaffia GPD* promoter (Verdoes et al., 1996) directly fused to the 211 bp 5'end of the Km^R gene, was generated using fusion PCR techniques (see below). The fragment was cloned in pUCG418, after digestion with *Kpn*I and *Msc*I, resulting in pPR1. A 3 kb *Sst*I *Phaffia* ribosomal DNA fragment (CHAPTER 3) comprising the 18S rDNA was cloned in the *Sst*I site of pPR1, resulting in pPR2. The *Phaffia* GPD-terminator was cloned downstream the Km^R gene in pPR2 as follows: The 281

bp *Phaffia* GPD terminator (Verdoes et al., 1996) was amplified by PCR (see below). The upstream oligo was chosen such that the nucleotides on position 5 (T) and 8 (T) were changed into A and C creating a *Bam*HI site at the 5'end. Furthermore, the downstream oligo contained a *Hind*III site. Thus a PCR generated *GPD* terminator DNA fragment was obtained flanked by a *Bam*HI and a *Hind*III site. This fragment was cloned downstream of the Km^R gene in pPR1, yielding pPR2T.

Polymerase chain reaction (pcr)

Standard reactions were carried out in an automated thermal cycler (Perkin-Elmer). Conditions: 5 min. 95°C , followed by 25 repeated cycli ; 2 min. 94°C, 2 min. 56°C, 3 min. 72°C. Ending by 1 cycle; 10 min. 72°C. Unless stated otherwise, plasmids pPRGDH6 (Verdoes et al., 1996) and pUCG418 were used as a template for generating the *Phaffia* GPD promoter/terminator and Km^R sequences respectively. Oligo's used (***Note:*** restriction sites are underlined, overlapping sequences bold, base substitutions are iticalized);

Generation of the 385 bp 3'part of the *Phaffia* GPD promoter, reaction 1;

*Hind*III *Xho*I *Kpn*I
1. 5'- CCCAAGCTTCTCGAGGTACCTGGTGGGTGCATGTATGTAC -3'

2. 5'- **TTCAATCCACAT**GATGGTAAGAGTGTTAGAGA -3'

Generation of the 211 bp 5'end of the Km^R gene, reaction 2;
3. 5'- **CTTACCATC**ATGTGGATTGAACAAGATGGAT -3'

*Msc*I
4. 5'-GCGTGACTTCTGGCCAGCCACGATAGC-3'

Generation of the 281 bp *GPD* terminator flanked by a *Bam*HI and *Hind*III/*Xho*I sites;

*Bam*HI
5. 5'-CCAAGGCCTAAAACGGATCCCTCCAAACCC-3'

*Hind*III *Xho*I
6. 5'-GCCAAGCTTCTCGAGCTTGATCAGATAAAGATAGAGAT-3'

Fusion PCR: A 0.6 kb PCR fragment, containing the 385 bp 3'part of the *Phaffia GPD* promoter directly fused to the 211 bp 5'end of the Km^R gene flanked by the *Hind*III, *Xho*I, *Kpn*I sites at its 5'end and *Msc*I at its 3'end, was obtained by adding the products from reaction 1 and 2 in equimolar amounts in a standard PCR reaction with oligo's 1 and 4.

Electro-transformation of *Phaffia*

Transformation of *Phaffia* strain CBS was performed based on an electroporation procedure as previously described (Faber et al., 1994), with following modifications: *Phaffia* was cultivated at 21°C in YPD medium (1% Yeast extract, 2% Bacto-peptone, 2% glucose) to an optical density at 600 nm of 1.2. Electropulsing was performed using the Bio Rad

Gene Pulser with Pulse Controller. No more than 5 µl of plasmid DNA were added to 60 µl of cell suspension in Bio Rad 0.2-cm cuvettes. Following the electric pulse (4 kV/cm, 1000 Ω, 25 µF) 0.5 ml YPD medium was added. The mixture was incubated for 2.5 h at 21°C and subsequently spread on selective YPD agar plates containing 40 µg/ml G418. Transformants were quantified after 5 days of growth.

Contour-clamped homogeneous electric field electrophoresis (CHEF)

Separation of high molecular weight DNA digests in 1% agarose was carried out using the Gene Navigator™ system (Pharmacia-LKB) in continuously circulating 0.5 x TBE buffer (50 mM Tris/borate; 1 mM EDTA) at 10°C. Electrophoretic conditions: 300 V with a switching time of 5 s during 5 h.

RESULTS

Transformation of linearized plasmid pPR2T to *Phaffia* by electroporation

Previously we found hcn integration in *Phaffia* using a plasmid containing the transposon Tn5 encoded Km^R gene fused in frame to the 5'terminal coding portion of the *Phaffia* actin gene, driven by the *Phaffia* actin promoter (CHAPTER 3). This plasmid also contained a 3 kb homologous rDNA portion to mediate integration in the rDNA. Transformants, that carried the plasmid at high copynumber in the rDNA, were obtained at very low frequency, despite earlier findings that the use of rDNA sequences in an integrative system significantly increases transformation efficiencies (Szostak and Wu, 1979). We assumed that the poor resistance of the transformants to G418 was the main reason for the low transformation efficiency. Since we pursued high efficiency transformation in the current investigation, we decided to use the *Phaffia* GPD promoter and terminator, previously cloned by Verdoes et al. (1996), to ensure high expression of the Km^R gene on the transcriptional level. It is established in other yeasts that glycolytic genes are constitutively highly expressed.

Plasmid pPR1 (FIG. 1) was constructed containing the *Phaffia* GPD promoter directly fused to Km^R gene. A 3 kb rDNA fragment from *Phaffia* (CHAPTER 3) containing the 18S and 5.8 S genes, was cloned in the SstI site of pPR1 resulting in pPR2. In addition it was decided to clone the *Phaffia* GPD terminator downstream the Km^R gene in pPR2, yielding plasmid pPR2T

Transformation of *Phaffia* was performed by electroporation mainly according to Faber et al. (1994) with modifications (MATERIALS AND METHODS).

Prior to transformation the plasmid was linearized in the unique ClaI site located in the rDNA portion to facilitate integration into the rDNA of the host.

The optimal conditions for electroporation were determined by monitoring transformation efficiencies at variable electric field strength and internal resistance values, whilst the capacitance was kept constant at 25µF (FIG. 2).

Average transformation efficiencies of approximately 1000 transformants per µg of ClaI linearized plasmid pPR2T DNA were obtained at an electric field strength of 4 KV/cm and an internal resistance of 1000 Ω. No transformants were obtained using circular plasmid suggesting the absence of an autonomous replication sequence (ARS).

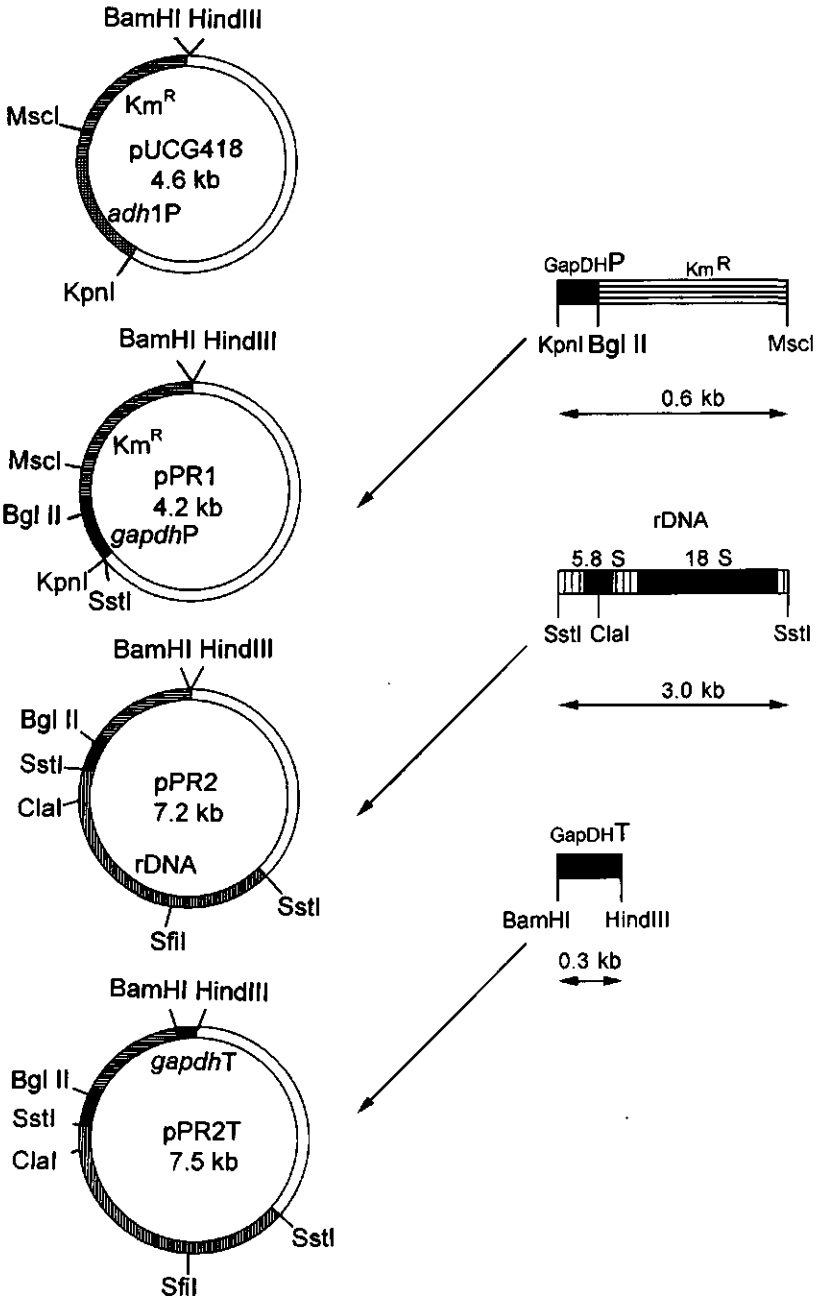

FIG. 1.

Schematic diagram of the plasmid construction for *Phaffia* transformation.

Boxes: empty, pUC18; horizontal stripes, Km^R gene; vertical stripes, rDNA; grid, *S. cerevisiae ADH1* promoter; black, *GPD* promoter/terminator.

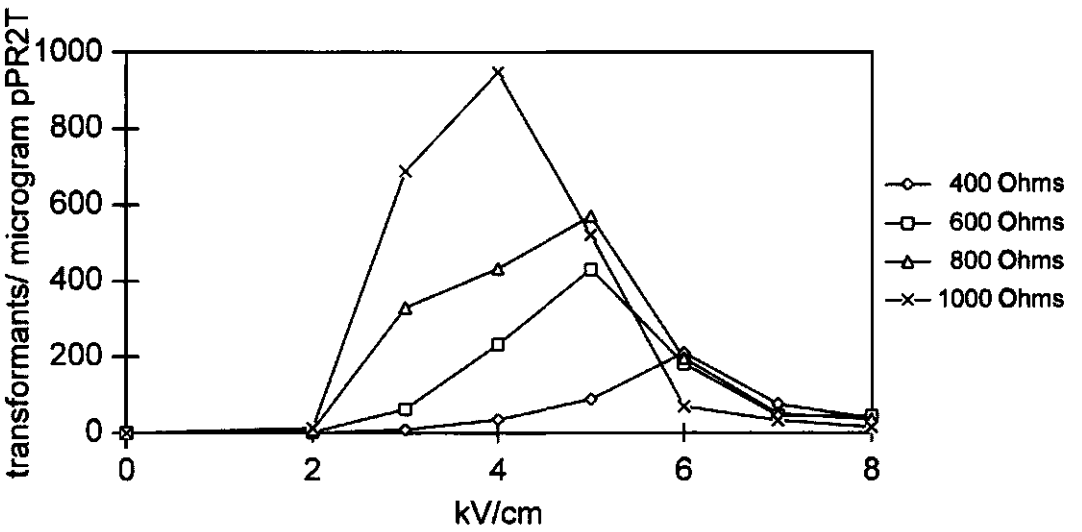

FIG. 2.
Influence of increasing electric field strength at different internal resistance's on transformation efficiency of *Phaffia*. In the legend the various internal resistance values are given in Ohms (Ω).Electric pulses were applied at a capacitance of 25 µF.

Influence of linearization site on transformation

Transformation efficiencies were influenced by the site used for plasmid linearization prior to transformation. After linearization at different unique sites in the rDNA portion (*Cla*I, *Sfi*I) of pPR2T, it was found that transformation with *Cla*I linearized plasmid yielded most transformants (1000/µg). A lower efficiency (180/µg) was obtained using *Sfi*I linearized plasmid (TABLE 1).

The transformants that were obtained using plasmid linearized with *Cla*I and *Sfi*I showed a high resistance to G418 (up to 2 mg/ml). In addition, all transformants were shown to be stable in that they showed normal growth on agar medium containing 200 µg/ml G418 after 60 generations of cultivation under non-selective conditions (TABLE 1).

Total DNA from three independent colonies transformed with the differently linearized pPR2T DNA's was isolated in order to determine the plasmid copy number. After digestion with *Bam*HI, which is uniquely present in the plasmid, the DNA's were separated by gel electrophoresis followed by Southern analysis using the 3 kb rDNA as a probe.

The DNA's analyzed showed a similar pattern of 4 hybridizing bands sizing 9, 8.5, 7.5 and 6.5 kb (FIG. 3 A). In FIGURE 3 A the results obtained with 3 *Phaffia* strains transformed with *Cla*I linearized plasmid are depicted (lanes 2, 3 and 4). The most intense band (8.5 kb) represents the hosts rDNA repeat, which consists of 61 rDNA copies (CHAPTER 3). The fainter hybridizing band of 7.5 kb represents integrated plasmid. Since *Bam*HI was not used as the linearizing enzyme prior to transformation it can be concluded that the plasmid copies are integrated in tandem (FIG. 3 D).

Comparison of the intensities of both the 8.5 and 7.5 kb band by scanning the autoradiogram indicates that on the average 20 plasmid copies are integrated (FIG. 3 A). The

TABLE 1. Influence of different transforming DNA's on the transformation efficiency, copy-number, G418 resistance and stability

	Plasmid: pPR2T Linearizing enzyme [e]			Plasmid: pPR2(-Term) Linearizing enzyme	
	none	*Cla*I	*Sfi*I	*Cla*I	*Sfi*I
Transformation efficiency [a]	0	1000	180	100	20
Average Copy number [b]	-	20	20	65	65
G418-resistance (mg/ml) [c]	-	2	2	2	2
Stability [d] (60 generations)	-	+	+	+	+

[a] Number of transformants per µg transforming plasmid
[b] Approximate number of copies of the transformed plasmid
[c] Maximum G418 concentration at which growth occurred
[d] Resistance to 200 µg/ml G418 after 60 generations of non-selective growth
[e] Enzyme used for linearizing plasmids pPR2T and pPR2 in the rDNA portion before transformation
- Term. = plasmid lacks the *GPD* terminator downstream of the Km^R-gene

hybridizing bands at approximately 9 and 6.5 kb represent DNA stretches consisting of a part of the two outer plasmid copies in the integrated stretch with flanking host rDNA sequences (FIG. 3 D). In FIGURE 3A, lane 4, an extra hybridizing band of approximately 6 kb is present, which may represent a pPR2T copy integrated outside the rDNA cluster.

Absence of the GPD terminator behind the Km^R gene reduces transformation efficiency and stimulates hcn integration

The influence of the *GPD* terminator on the transformation efficiency and plasmid copy number was investigated. Using *ClaI* and *SfiI* linearized plasmid pPR2, which lacks the *Phaffia GPD* terminator downstream of the Km^R gene, transformants were obtained with a significant lower average frequency of 100 and 20 per µg plasmid DNA respectively. A 10-fold reduction compared to the transformation frequency obtained with plasmid pPR2T was observed (TABLE 1).

Similar to the transformants obtained with pPR2T, pPR2 transformants were resistant to up to 2 mg/ml G418 and maintained their resistance to G418 after 60 generations of non-selective growth.

Total DNA obtained from three independent pPR2 transformed colonies was digested with *Bam*HI. Following agarose gel electrophoresis, Southern analysis on the digestion pattern was performed using the 3 kb SstI rDNA fragment as a probe (FIG. 3 A, lanes 5-7). It can be deduced, by comparison of the hybridization intensities of the bands representing the multiple rDNA units (8.5 kb) and the plasmid (7.5 kb), that on the average 65 copies of plasmid pPR2 were present in these transformants.

These results indicate that the absence of the *GPD* terminator downstream of the Km^R gene causes a sharp decrease of transformation efficiency, whereas the plasmid copy number is on the average increased of by a factor 3. Apparently the *Phaffia* GPD terminator plays an important role in the expression of the KmR resistance gene, which is

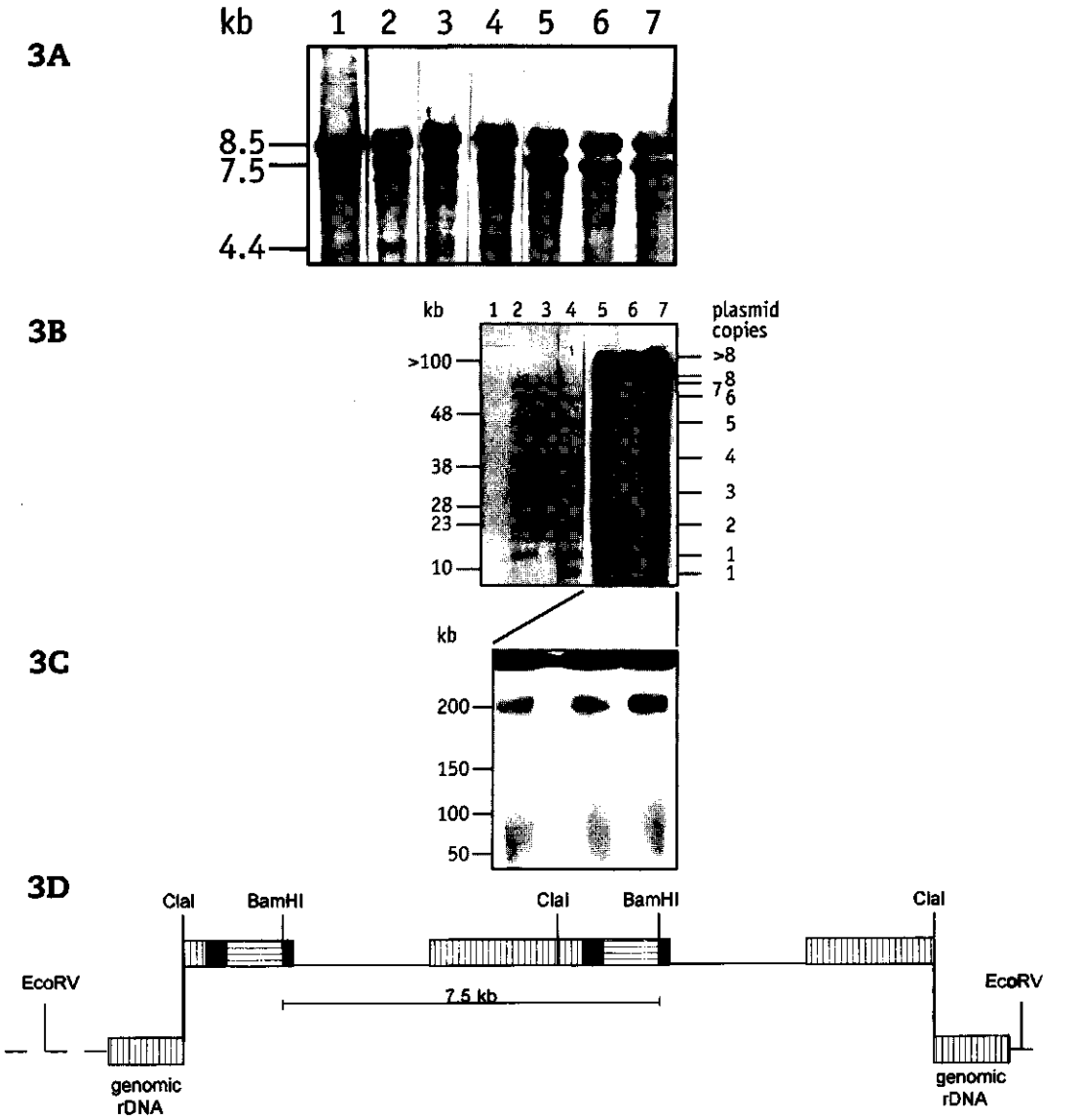

FIG. 3.

Presence of plasmid in *Phaffia* strains transformed with plasmid pPR2T or pPR2 as analyzed by Southern hybridizations using the DIG DNA Labeling and Detection Kit (Boehringer). **(A)** shows the hybridization of *Bam*HI digested total DNA after agarose gel electrophoresis from wild type *Phaffia rhodozyma* (lane **1**), *Phaffia* pPR2T transformants I, II and III (lanes **2-4**) and pPR2 transformants **I, II** and **III** (lanes **5-7**), with a 3 kb *Sst*I *Phaffia* rDNA fragment as a probe. **(B)** differs from **(A)** in that *Eco*RV digested total DNA was used for hybridization with pUC18 as a probe. **(C)** shows the hybridization of *Eco*RV digested total DNA from *Phaffia* pPR2 transformants I, II and **III** after separation by means of countour-clamped homogeneous electric field electrophoresis (CHEF). **(D)** A schematic map, illustrating that multiple copies of plasmid are tandemly integrated in the genomic rDNA of Phaffia transformants. The linearizing enzyme *Cla*I and the enzymes used for hybridization studies (*Bam*HI and *Eco*RV) are depicted. Boxes: horizontal stripes, Km^R gene; vertical stripes, rDNA; black, *GPD* promoter/terminator.

in agreement with the well established fact that the presence of a homologous terminator may increase the expression of foreign genes in that mRNA 3'end formation occurs more efficient, contributing to more stability of the mRNA (Raué, 1994).

Plasmid DNA is integrated at multiple sites in tandem arrays that contain maximally 30 plasmid copies

It was shown by Lopes et al. (1991) that plasmids targeted to the rDNA of *S. cerevisiae* may integrate at different sites in the rDNA repeat. It was shown that plasmid DNA was mainly integrated tandemly in one large cluster. In addition, the presence of smaller plasmid repeats containing 1-3 copies was demonstrated.

In order to investigate the distribution of integration sites of *Phaffia* transformants and the manner in which the multiple plasmid copies are integrated, total DNA from 3 transformants, containing either pPR2 or pPR2T DNA, was digested with enzymes that do not cut the plasmids, but do cut the rDNA unit of *Phaffia*. In this manner DNA fragments containing one or more plasmid copies are excised (**FIG. 3 D**).

The digested DNA was separated by contour-clamped homogeneous electric field electrophoresis (CHEF) and southern analysis was performed using pUC18 DNA as a probe (**FIG. 3 B**). In the lanes 2, 3 and 4, that contain *Eco*RV digested DNA from *Phaffia* strains transformed with pPR2T a ladder of maximally 8 hybridizing DNA fragments is visible, each ascending rung of the ladder representing increasing plasmid copies. In lane 4 again the single integration event outside the rDNA is visualized by an extra hybridizing band of approximately 10 kb. Similar results were obtained with *Sal*I digested DNA's (not shown).

It appears that this ladder-like hybridization pattern represents a heterogeneous population of transformant cells with different integration patterns, rather than a homogeneous population carrying up to 8 dispersed clusters that only differ one plasmid copy in length. This is sustained by two observations. First, the hybridization signal intensity does not increase with plasmid copy number. Second, the total number of plasmid copies, as deduced by the summation of hybridizing fragments in **FIGURE 3 B**, differs from the plasmid copy number as deduced from signal intensity comparison (**FIG. 3 A**). PPR2T Transformants I, II and III, were estimated to carry 24, 20 and 16 plasmid copies respectively by comparison of hybridization signal intensities (**FIG. 3 A**), whereas 36, 36 and 28 plasmid copies respectively are predicted by adding the plasmid copynumbers represented by the hybridizing bands (**FIG. 3 B**).

In conclusion plasmid pPR2T is integrated in tandem arrays of 1 to 8 copies, that are located at different sites in the host's rDNA repeats.

Phaffia transformants that carry the terminator lacking plasmid pPR2 DNA differ from pPR2T transformants in that the plasmid is mainly integrated in arrays consisting of more than 8 plasmid copies (**FIG. 3 B, lanes 5-7**).

To further investigate the exact size of the largest integrated plasmid clusters in these transformants, chromosomal samples were prepared in low melting point agarose plugs as described previously (Nagy et al., 1994) to avoid DNA shearing. The DNA's were subsequently digested in the plugs with *Eco*RV or *Bst*XI, separated by means of contour-clamped homogeneous electric field electrophoresis (CHEF) and analyzed by Southern hybridization with pUC18 DNA as a probe. Both digestions gave rise to the predominant presence of two hybridizing DNA fragments of approximately 200 kb, indicating that in these transformants plasmid pPR2 is integrated mainly in two tandem repeats each

containing approximately 30 copies. In FIGURE 3C only the EcoRV digested DNA's are shown.

The G418 resistance of transformed *Phaffia* cells increases during long term incubation without selective pressure: indications for selection pressure independent plasmid amplification in the *Phaffia* rDNA

In our transformation experiments we applied a selection pressure sufficient to eliminate background growth, using YPD agar plates containing 40 µg/ml G418. We found that many transformants, both with plasmid pPR2 and pPR2T, selected on these plates grew well on plates containing 2 mg/ml G418.

A possible explanation for this event could be that in response to the elevated selection stress the cells rapidly increase the plasmid copynumber. It was previously shown, that hcn integration in the rDNA of *S. cerevisiae* (Lopes et al., 1991), *Kluyveromyces lactis* (Bergkamp et al., 1992) and *Yarrowia lipolytica* (Le Dall et al., 1994) correlated with a weak expression of the marker gene by a truncated promoter.

However, in our system the marker is driven by an intact glycolytic promoter suggesting a different mechanism. Furthermore, despite the fact that the analyzed *Phaffia* transformants were never exposed to G418 concentrations higher than 40 µg/ml, they carried both pPR2 and pPR2T at high copynumbers (FIG. 3). This observation indicated that the transformed cells may actually carry more plasmid copies than strictly required for survival on the selection plates and that hcn integration in the *Phaffia* rDNA may occur independently from selection pressure.

To provide further insight in this matter we investigated whether transformed *Phaffia* cells gain a higher resistance to G418 after prolonged non-selective incubation. For this purpose pPR2T and pPR2 DNA was transformed to several batches of *Phaffia* cells (FIG. 4). After electropulsing both types of cell/DNA mixtures were immediately pooled in non-selective YPD medium yielding two pools of cells able to form 20.000-50.000 colonies on selective agar medium (40 µg/ml G418). From these pools aliquots were spread onto plates containing increasing concentrations of G418 (40-2000 µg/ml) at several intervals during 30 hrs of incubation after electropulsing. Colonies were quantified after 5 days of growth. This experiment was repeated twice independently with a similar outcome. The average values are shown in FIGURE 4.

In FIGURE 4 A and B the fractions of the total number of pPR2 and pPR2T transformant colonies that grew on the different G418 concentrations are depicted at each interval. In FIGURE 4 C the average G418 resistance per transformant colony is derived.

These results show that the distribution of subpopulations of both pPR2 and pPR2T transformed cells with different resistances to G418 changes under non-selective conditions towards subpopulations with higher resistances (FIG. 4 A+B). This results in a 3 fold higher average G418 resistance (FIG. 4 C). In the first 5 hours of incubation the resistance of pPR2T transformants increased significantly more than pPR2 transformants, indicating a higher expression of the terminated Km^R-gene.

During the experiment the growth of the *Phaffia* cells (non-transformed + transformed) in both pools was monitored by spreading dilutions on non-selective YPD plates at different time intervals. Between 0 hr and 5hr of incubation the number of colonies only slightly increased, indicating that the cells resume growth within 5h after electropulsing. Between t=5h and t=23h a 50 fold increase took place (not shown), whereas after

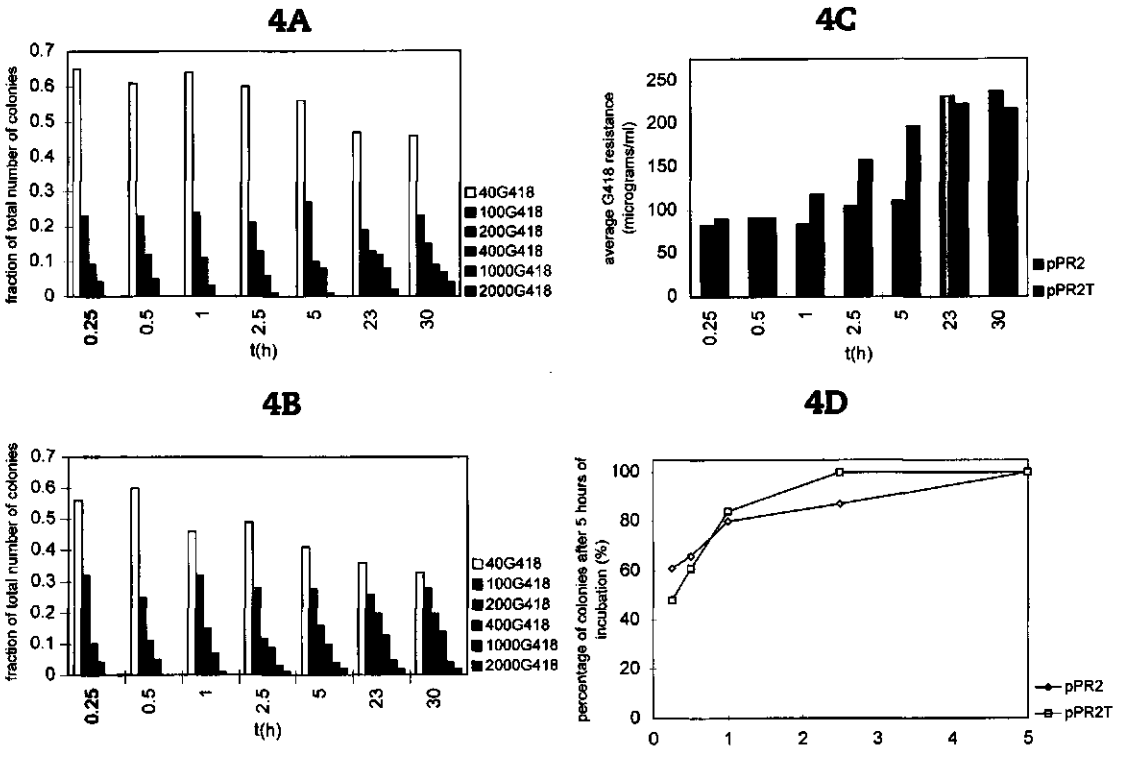

FIG. 4.

Shift to *Phaffia* transformants with higher G418 resistances after prolonged non-selective incubation. 50 µg of *Cla*I linearized pPR2T (2.5 µg/µl) and 200 µg (2.5 µg/µl) of *Cla*I linearized pPR2 were transformed to 4 and 16 portions (60µl) of competent *Phaffia* cells, respectively. After electropulsing and resuspending in YPD both types of cell/DNA mixtures were pooled and instantly further diluted in YPD (20 fold). From both pools aliquots were taken after 0.25, 0.5, 1, 2.5, 5, 23, and 30 hrs of incubation and spread on YPD agar plates containing 40, 100, 200, 400, 1000 and 2000 µg/ml G418. After 5 days of growth colonies were quantified. In **(A)** and **(B)** the results obtained with pPR2 and pPR2T respectively are depicted. The bars represent the fraction of the total number of colonies on a specific G418 concentration, at each time interval. In **(C)** the average resistance, expressed as the sum of the fraction of colonies x [G418], at each time interval of both pPR2 and pPR2T transformants is shown by the bars. **(D)** shows the increase of transformation efficiency in the first 5 hours of non-selective incubation. The number of colonies that appeared on YPD plates containing 40 µg/ml G418 was quantified after 0.25, 0.5, 1. 2.5 and 5 hrs of incubation. In the chart the transformation efficiency is expressed as percentage of the number of colonies at 5 h.

t=23h growth stopped. The number of transformant colonies, growing on YPD + 40 µg/ml G418, also increased after prolonged non selective incubation (**FIG. 4 D**). At 2 stages a significant increase of transformant colonies was observed. Between t=0.25h and t=1h the number of pPR2 and pPR2T transformants increased by 30% and 70% respectively. Since this time frame was too short for growth, this can be explained by the recovery of the cells after electropulsing and the triggering of the transcriptional and translational machinery. The difference in recovery could be explained by a more efficient transcrip-

tion of the terminated marker gene in pPR2T. Between t=5h and t=23hr the number of transformants increased by a factor 10 - 20 (not shown), which can be ascribed to division of the transformant cells.

These results show that before, during and after growth, transformant cells gain higher resistances to G418, supporting the view that in the rDNA integrated plasmid copies amplify without selection pressure. The extend of amplification appears to be dependent on the plasmid used.

Discussion

In this report we show that efficient transformation of the basidiomycetous yeast *Phaffia rhodozyma* can be successfully performed using a plasmid that is targeted to the rDNA locus. Similar to other systems described for the ascomycetous yeasts *S. cerevisiae* (Lopes et al., 1989), *K. lactis* (Bergkamp et al., 1992), *Y. lipolytica* (Le Dall et al., 1994), stable hcn integration is obtained.

The mode in which hcn integration is obtained in the *Phaffia* rDNA appears to differ from these organisms. It was suggested that in *S. cerevisiae* hcn integration in the rDNA is dependent on the use of a marker gene (auxotrophic) driven by a truncated promoter (Lopes et al., 1991). We found hcn integration in *Phaffia* using an intact glycolytic promoter to drive expression.

It could still be argued that despite the use of this promoter, the expression of the Km^R gene is still poor, forcing plasmid amplification to a level sufficient for transformants to survive. The limited amplification in pPR2T transformants could hence be explained by presence of the terminator, enhancing a more efficient transcription of the Km^R gene.

However, we have shown that transformants that were exposed to the lowest G418 level (40 µg/ml) still show hcn integration. In addition, these transformants were also able to grow on G418 concentrations of up to 2 mg/ml, suggesting a selection pressure independent plasmid amplification.

Furthermore, it was shown that prolonged incubation (30 hr) of both pPR2 and pPR2T transformed cells in non selective medium gave rise to an average increase of G418 resistance by a factor 3 (Fig. 4C). It could be argued that the gain of G418-resistance in this experiment was rather due to the building of the transcriptional and translational machinery than to plasmid amplification. Two observations, however, are not in agreement with this view. First, the G418 resistance was shown to increase significantly during cell division at which stage expression is likely to be fully turned on. Second, the resistances to G418 of both pPR2 and pPR2T transformants, differing significantly in the first hours of incubation, became similar after 30 hrs. If the increased level of G418 resistance were solely to be attributed to an enhanced expression of a fixed number of integrated plasmid copies, pPR2T transformants would show a higher resistance to G418 than pPR2 transformants after 30 hrs of incubation.

Thus, the increase of G418 resistance appears to arise from amplification of plasmid copies in the genomic rDNA of *Phaffia*, independent from selection pressure.

From both pPR2 and pPR2T transformants circular and unmodified plasmid could be recovered after non-selective growth for over 10 generations by transformation of total

DNA to highly competent *E. coli* (not shown). Since both plasmids apparently lack ARS activity these circular forms could well originate from excision of integrated forms as a mechanism to counter-act amplification. Subsequently these circular forms may integrate at various locations in the rDNA cluster and be amplified, explaining the dispersed integration patterns in **FIGURE 3B**.

The picture emerges that the copynumber of plasmid pPR2T and pPR2 is a result of amplification and excision. As the copynumber rises, either the metabolic burden becomes disadvantageous to the cell or limitations occur, driving amplification and excision to an equilibrium.

Apparently this equilibrium is reached at a significant earlier stage in pPR2T transformants (20 copies) than in pPR2 (65 copies) transformants. This could be explained by the more efficient expression of the Km^R gene in pPR2T, leading to an enhanced increase of the metabolic burden as the plasmid copy number rises. In this context it should be noticed that the average resistance of both pPR2T and pPR2 transformants is similar. The extend to which amplification occurs within a pPR2 and a pPR2T transformant population is also variable. This is shown by both the differences in G418 resistance (**FIG. 4 A+B**) and the ladder-like hybridization patterns of in particular pPR2T transformants (**FIG. 3 B**). Apparently individual transformants show different levels of tolerance to the increasing plasmid amplification.

The transformation system for the astaxanthin producing yeast *Phaffia rhodozyma* we developed could serve multiple purposes. First, the system is highly efficient, which is useful for the isolation of (carotenoid) genes by complementation of defined *Phaffia* mutants. Second, for the overexpression of any gene, the manner in which the copy number is regulated in the *Phaffia* rDNA could be of value for establishing the proper ratio between expression levels and yield. Third, the transformation system is stable, which is a prerequisite for long term and large scale production.

6.
Astaxanthin production by *Phaffia rhodozyma*

Introduction

The results obtained in Chapter 5 show that the transformation system for *Phaffia* has promising features for the isolation of other *Phaffia* genes, which will contribute to a further genetic characterization. In addition *Phaffia* is now accessible for the use of recombinant DNA technology, which could ultimately lead to industrially improved strains.

This Chapter will discuss the use of *Phaffia* as a source of astaxanthin. In the first part the applications and commercial importance of astaxanthin, which was repeatedly and briefly referred to in the introductions of the foregoing chapters, will be more extensively reviewed. In addition, the use of recombinant DNA technology for obtaining commercially attractive astaxanthin overproducing *Phaffia* strains is contemplated with the current understanding of the biosynthesis of the astaxanthin biosynthesis pathway.

Use of astaxanthin in the fish farming industry

Astaxanthin is a red oxygenated carotenoid (xanthophyll) (Fig. 1) that is widely distributed in nature and synthesized *de novo* primarily by green algae.

Astaxanthin is traditionally used as a fish feed additive for salmon and trout. Like a range of other red and pink organisms, such as birds and crustaceans, salmonids deposit astaxanthin in the flesh upon ingestion and are incapable of *de novo* synthesis of the colorant (Simpson, 1982). Since the color of the flesh of these expensive fish is important for the appreciation by the consumer, the use of astaxanthin is an absolute requirement for marketing.

Both the significant increase in the production of pen-raised salmonids, from maximally 10.000 metric tons in 1975 to approximately 600.000 tons in 1991 (according to the FAO fisheries circular #815, 1991), and the increased farming of shrimp and lobster have contributed to a growing demand for astaxanthin.

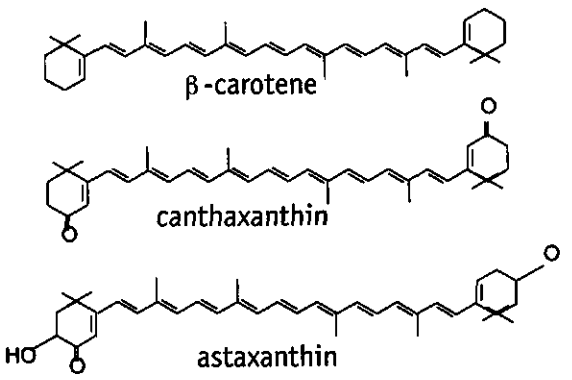

Fig. 1.
Molecular structure of β-carotene, canthaxanthin and astaxanthin

Most of the commercially available astaxanthin is produced chemically by Hoffmann-LaRoche and sold as water dispersible gelatin beadlets containing 5% pure astaxanthin for approximately $2400 US per kg. The use of astaxanthin as a feed supplement raises its cost by approximately 15% and therefore several investigations were carried out to establish economical carotenoid supplemented diets for salmon and trout (No and Storebakken, 1992; Smith et al., 1992; Bjerkeng et al., 1992). Most important factor for marketable salmonids is the coloration of the flesh as determined by vision. According to Torrissen (1989) approximately 4 mg astaxanthin per kg of flesh is acceptable. In addition astaxanthin concentrations higher than 6 mg/kg cannot be distinguished for by vision. Therefore, if the fading of flesh color by storage etc. is compensated for, salmon flesh should contain approximately 5 mg/kg astaxanthin.

It was shown for pan-size salmon that this value could be obtained by the use of 15 mg astaxanthin/kg feed, administered throughout the production cycle (28 weeks) (Smith et al., 1992). It is estimated for the fish farming industry in general that currently approximately 35 mg/kg of astaxanthin is used in the feed (Smith et al., 1992), which would imply that the market for synthetic astaxanthin for solely salmonid aquaculture accounts for roughly 100 million US dollars.

Canthaxanthin (FIG. 1) is also used by fisheries as a cheaper ($1300/kg) alternative for astaxanthin. However, it was shown for rainbow trout that astaxanthin was 1.5 times more efficiently utilized than canthaxanthin (Foss et al., 1984) by better binding of astaxanthin to the muscle actomyosin (Henmi et al., 1987 and 1989) and a faster metabolic turnover of canthaxanthin. Furthermore, the latter was shown to fade faster (Simpson et al., 1981). These properties question the use of canthaxanthin as a more profitable pigment source.

Astaxanthin and health

Apart from the fish farming industry the use of astaxanthin as a "functional food" is anticipated following a number of recent papers reporting a possible role in the prevention of cancer.

It has been established by epidemiological studies both in animals and in cell culture that carotenoids act chemopreventive on the development of cancer. It appears that this preventive action can be ascribed multiple factors, including pro-vitamin A activity (reviewed by Weisburger 1991), inhibition of cancer cell proliferation (Zhang et al., 1992), radical-trapping activity (antioxidant) (Palozza and Krinsky, 1992; Lim et al., 1992), and immune system stimulation (Jyonouchi et al., 1995). The anti-tumor action of β-carotene (FIG. 1) has been extensively described and is presumed to arise from its provitamin A activity.

Following indications that xanthophylls as a dietary supplement could lower the risk of cancer, several studies were initiated concerning the role of astaxanthin and canthaxanthin in the chemoprevention of carcinogenesis in mouse (Tanaka et al., 1994) and rat (Tanaka et al., 1995), prevention of lipid peroxidation in membrane models (Palozza and Krinsky, 1992; Lim et al., 1992) and immune system stimulation (Jyonouchi et al., 1995).

A striking difference between β-carotene and astaxanthin was observed in these studies. It was shown that, contrary to β-carotene (Moon, 1989), astaxanthin and canthaxan-thin suppressed experimental bladder carcinogenesis in mice (Tanaka et al., 1994). Furthermore, it was claimed that astaxanthin had a greater inhibitory effect on

tongue tumorigenesis in rat than β-carotene (Tanaka et al., 1995).

Since astaxanthin (and canthaxanthin) have no pro-vitamin A activity their anti-tumor effect are probably due to other properties. Firstly it was shown by Zhang et al. (1992) that both β-carotene and canthaxanthin could increase the expression of the mouse connexin43 gene, thereby stimulating gap junctional intracellular communication, which inhibits cancer cell proliferation (Zhang et al., 1991; Hossain et al., 1989). Secondly, several studies have shown that astaxanthin is a much more potent antioxidant than β-carotene. Palozza and Krinsky (1992) found that astaxanthin and canthaxanthin could trap radicals in biological membranes as effectively as the classical antioxidant α-tocopherol, whereas β-carotene had much lower antioxidant activity. Furthermore, Lim et al. (1992) found that higher levels of astaxanthin in the plasma of chicks could compensate for decreased levels of α-tocopherol with respect to antioxidant defense. β-carotene was shown not to compensate for the loss of α-tocopherol.

Finally Jyonouchi et al. (1995) showed that astaxanthin enhanced immunoglobulin production by human peripheral blood mononuclear cells in *in vitro* experiments. It was also shown that β-carotene did not have a significant effect.

In 1992 β-carotene underwent clinical trial as a cancer preventive agent in the US (Malone, 1991).

In view of the promising properties of xanthophylls like astaxanthin for human health, the use of this natural compound as a therapeutic agent and as an supplement in food can be anticipated, which will result in a growing market for these compounds.

Natural sources of astaxanthin and their potential for commercial use

1. *Krill and crustacean waste*

Alternative microbiological sources of astaxanthin that could economically compete with the synthetic form have been evaluated.

Storebakken (1988) reviewed the use of krill as a potential feed source for salmonids. Krill constitutes of approximately 85 species of small marine crustacean that contain various forms of astaxanthin as the main carotenoid (15-77 mg/kg), whilst in krill meal and krill oil these values vary from 15-200 (Kotik et al., 1979; Quantz, 1980; Yamaguchi et al., 1983) and from 700-1000 mg/kg (Lambertsen and Braekkan, 1971; Fujita et al., 1983), respectively. Krill can only be used as a feed supplement, since the use of krill as a sole feed stuff for salmonids resulted in reduced growth. For satisfactory growth maximally 20% of krill can be used in moist feed, resulting in a total astaxanthin content of approximately 20 mg/kg. However, the major part of krill astaxanthin is esterified with fatty acids to form mono and diesters. These forms were shown to be utilized by salmonids to a much lesser extend than the free astaxanthin form (Storebakken, 1988). As a result the adding of extra synthetic astaxanthin is a prerequisite for proper coloration of salmonids on a krill containing diet.

Shrimp and other crustacean by products have also been regarded as potential natural astaxanthin sources. The annual wild-catch of shrimp from 1982-1992 was approximately 1.8 million metric tons (Rosenberry 1993), providing an enormous quantity of astaxanthin containing processed waste. However, of approximately 35 mg/kg of total astaxanthin that is present in shrimp waste, only 5% is in the free form (Guillou et al., 1995). If shrimp waste were to be added to 25% of the feed the final free astaxanthin content would be far too low (0.4 mg/kg) for acceptable salmonid pigmentation.

2. The alga *Haematococcus pluvialis*

In the past several astaxanthin producing organisms, ranging from marine bacteria (*Agrobacterium aurantiacum*) (Yokoyama et al., 1994) and algae (*H. pluvialis*, *Neochloris wimmeri*, *Chlamydomonas nivalis*) (Tischer, 1938; Brown, 1967; Czygan, 1986) to fungi (*Phaffia*) (Phaff et al., 1972), have been isolated. Many of them synthesize astaxanthin as the minor carotenoid in low amounts. However, the green alga *H. pluvialis* and the basidiomycetous yeast *Phaffia rhodozyma* are currently used on the industrial scale for astaxanthin production.

The algae belonging to the genus *Haematococcus* have been a subject for investigation since 1907 mainly because of their remarkable cell-cycle and cell structure. The organism can adopt the form of a small green motile biflagellate cell, 4 to 5 µm in diameter (Boussiba and Vonshak 1991), with the plasma membrane only connected to the cell wall through cytoplasmic strands (Bubrick 1991). Under stress conditions, such as N-limitation, salt stress (1% NaCl), phosphate starvation and high light intensities, the morphology changes in that a big round immotile cell sizing 15-20 µm with a thick persistent wall is formed (Boussiba and Vonshak 1991). Under favorable conditions these so called cysts can germinate and release individuals of the motile form.

The cyst stage of *H. pluvialis* is also characterized by the formation of large amounts of carotenoids (up to 5% w/w) of which different forms of astaxanthin are predominant (80%) (Grung et al., 1992). This feature has led to the interest of industry for this organism and to several investigations concerning optimization of astaxanthin production.

The role of astaxanthin formation in *H. pluvialis* is not quite understood. Although it had been shown that accumulation of carotenoids in other algae prevented damage to the photosynthetic apparatus by high radiation, Boussiba and Vonshak (1991) rejected the hypothesis of such protective role in *H. pluvialis*. They argued that astaxanthin in *H. pluvialis* is not localized in the chloroplasts and the production of astaxanthin can also occur in the dark. In addition, they found that under astaxanthin production promoting conditions, *H. pluvialis* lost most of its photosynthetic activity.

The use of *H. pluvialis* astaxanthin as a feed ingredient in aquaculture for the coloration of salomonids was evaluated by several groups. Grung et al. (1992) showed that in the alga just over 1% of the astaxanthin is present in the free form, whereas 42% and 57% is in the form of diesters and monoesters, respectively. These forms are less well utilized by salmonids (Storebakken et al., 1987) requiring higher amounts of the algal carotenoid for proper coloration. Furthermore, Sommer et al. (1991) showed that the use of dried whole *H. pluvialis* cysts reduced the bioavailability of the astaxanthin to the fish, probably due to the resistance of the tough cell wall to digestion.

In feeding trials conducted by Sommer et al. (1992) it was established the use of algal powder (1,9% total carotenoid) as a fish feed ingredient could lead to rather satisfactory trout flesh astaxanthin levels (2.37 mg/kg using 80 mg algal astaxanthin/kg feed with respect to 5 mg/kg using 80 mg/kg synthetic astaxanthin). This result, however, could only be reached after the algal biomass was subjected to several processing steps, like breaking of the cysts cell wall, homogenizing and spray-drying.

Astaxanthin from *H. pluvialis* is commercially produced as a stabilized processed powder (Algaxan Red) by Microbio Resource Inc. San Diego. One kg of algal astaxanthin sells for $2000, which is comparable to the price of the synthetic compound. However, the findings that the latter is deposited 40-45% more effective in trout flesh (Sommer et al., 1992) may indicate that algal astaxanthin at this stage is still too expensive for economic competitiveness.

3. *Phaffia rhodozyma*

In addition to *H. pluvialis*, the red basidiomycetous yeast *Phaffia rhodozyma* is also regarded as a microbiological source of astaxanthin that could be commercially attractive.

Phaffia can grow both aerobically and anaerobically and is able to synthesize up to 400 µg of astaxanthin per gram dry weight as the main carotenoid under aerobic conditions in the late log phase. Whilst *H. pluvialis* synthesizes the (3S, 3'S)-optical isomer of astaxanthin (Grung et al., 1992) and the synthetic carotenoid consists of a mixture of the (3S, 3'S)-, (3R, 3'S)- and (3R, 3'R) isomers (Bjerkeng et al., 1992), *Phaffia* synthesizes the (3R, 3'R)-isomer and unlike in *H. pluvialis* all astaxanthin is produced in the free form and is shown to have a protective role.

It was suggested by Schroeder and Johnson (1993) that the carotenoids in *Phaffia* compensated for the lack of both high levels of catalase in the stationary phase and cytosolic super oxide dismutases as reactive oxygen species detoxifiers. They showed that under O_2-generating conditions the total carotenoid content as well as the relative astaxanthin amount increased in *Phaffia*. It was also shown by these authors (1995) that the use of singlet oxygen was effective in selecting for astaxanthin (hyper) producing *Phaffia* strains.

The natural habitat of *Phaffia* is on slime fluxes of deciduous trees (birch) at high elevations. The ecological rationale for carotenoid accumulation in *Phaffia* was elucidated, after it was shown that birch tree exudate contains a substance that catalyzes the formation of singlet oxygen upon UV radiation.

Phaffia has long been regarded as a potential economically competitive producer of astaxanthin due to some favorable properties. First, this yeast is easy to cultivate to high cell densities on cheap undefined substrates like molasses. Second, all astaxanthin is produced in the free form, and well utilized by salmonids (Binkowski et al., 1993). However, the low astaxanthin content of wildtype strains (up to 400 µg/g DW) has been a drawback. Several strategies have been applied to increase astaxanthin levels, including optimization of growth substrates, protoplast fusion and classical mutagenesis. It was found that astaxanthin production could be enhanced by the addition of precursors of its biosynthesis route, like acetic acid (Meyer and du Preez, 1993), mevalonic acid (up to 4x wt level) (Calo et al., 1995), monoterpenes (Meyer et al., 1994) as well as tomato waste (Johnson and Lewis, 1979). For large scale fermentations, however, addition of these substrates would be less suitable, due to costs, or declining cell yields.

The isolation of *Phaffia* strains with increased astaxanthin content proved to be more promising. To achieve this Chun et al. (1992) applied protoplast fusion. Hybrid strains were obtained that produced 2000 µg/g.

Other groups were successful using classical mutagenesis. An et al. (1989) reported that the use of antimycin A, an inhibitor of electron transport, in agar plates was successful for the isolation of astaxanthin overproducing strains (900 µg/µl). Further mutagenesis with NTG led to isolates containing approximately 1500 µg/g.

A different selection method was presented by Lewis et al. (1990). They plated NTG mutagenized *Phaffia* cells on plates with β-ionone as a selective agent. This compound is an analog of the end ring of β-carotene and as such probably competes for the oxygenation of β-carotene to form astaxanthin. Wildtype *Phaffia* cells therefore have a pale color on these selection plates, whereas astaxanthin overproducing mutants are visible as red colonies.

Using these methods, with several mutation rounds, astaxanthin overproducing

strains were isolated (Lewis et al., 1990; Fang and Cheng, 1993; Meyer et al., 1993) that contained up to 2500 µg/g astaxanthin.

It is generally recognized that astaxanthin over-producing *Phaffia* mutants are susceptible to reversion necessitating multiple rounds of mutagenesis. This easily leads to isolates that are cripple in that cell yields and growth rates decrease, which often leads to lower astaxanthin yields. However, Fang and Chiou (1996), showed that mutagenesis of *Phaffia* cells followed by optimization of growth conditions in batch cultivation can lead to overproducing mutants with a relatively high volumetric astaxanthin production of 16.33 mg/l, whereas typical values of 2 mg/l could be obtained with wildtype *Phaffia* (Johnson and Lewis 1979). In addition, Johnson and Schroeder reported that Universal Bioventures cultivates a *Phaffia* strain, on a commercial scale, producing 4000 µg/g astaxanthin.

Currently, stabilized *Phaffia* powder is produced by Universal Foods (USA), containing approximately 3000 µg/g astaxanthin.

The use of *Phaffia* astaxanthin as a pigment source for rainbow trout was evaluated by Binkowski et al.(1993). They found that the use of 10% (w/w) whole *Phaffia* yeast in the fish diet (67 ppm astaxanthin) gave rise to normal weight gain and a coloration of the flesh as satisfactory as the use of comparable amounts of synthetic astaxanthin. Opposed to *H. pluvialis*, the digestibility of the *Phaffia* cell wall proved to be satisfactory, lifting the need for costly mechanical or enzymatic disruption procedures. Furthermore, it was shown by Nakano et al. (1995) that use of *Phaffia* as a dietary supplement contributed to the improvement of the overall fish-health indicating that a large fraction of the fish feed may consist of whole *Phaffia*, without affecting the fish yield.

Despite these favorable features, the use of *Phaffia* as an astaxanthin source is still relatively limited, because, like other alternative astaxanthin sources, the production of the colorant is still too expensive. It is estimated that at least a 10 fold increase of the wildtype astaxanthin production in *Phaffia* would be needed to compete with the chemical production at its current market price.

It appears that conventional improvement strategies like random mutagenesis have reached their limits with commercially exploited *Phaffia* strains that produce 3000 to 4000 µg/mg. The use of recombinant DNA technology in *Phaffia* research could be an option in order to obtain higher productivity. One should, however, first investigate in what way gene technology can be of help to improve astaxanthin biosynthesis. This is complicated by the fact that this synthesis involves many enzymatic reactions encoded for by various genes that are differently regulated. The simple overexpression of a particular gene would be in most cases useless, unless it encodes a "bottle neck" enzyme or plays a key regulatory role. Proper knowledge of the astaxanthin biosynthesis pathway is therefore important. The use of recombinant DNA techniques (development of a transformation system and the isolation of genes), should in the first place provide information on the genetics of carotenoid production in *Phaffia*. In a later stage this knowledge can be used for the overproduction of astaxanthin.

The biosynthesis of astaxanthin

Introduction

The biosynthesis of astaxanthin starts with the mevalonate (MVA) pathway (Fig. 2). This pathway involves soluble enzymes to produce isoprenoids that are precursors for many

vital compounds like sterols, steroid hormones, lipoproteins and vitamin D.

The carotenoid biosynthesis route, that involves membrane bound enzymes, branches of from the MVA pathway at farnesyl pyrophosphate and proceeds into the xanthophyll (oxygenated and hydroxylated carotenoids) biosynthesis. Whereas the biosynthetic conversions in the MVA pathway are similar through diverging species, the number of different carotenogenic compounds appears to increase overproportional descending the carotenoid/xanthophyll pathway as the many carotenoid producing microorganisms and plants have obtained numerous ways to introduce oxygen and hydroxyl groups to into carotenes.

Several reviews have discussed the isoprenoid (Kleinig 1989; Goldstein and Brown 1990; Randall et al., 1993) and carotenoid biosynthesis pathway (Bramley and Mackenzie, 1988; Sandmann, 1994; Armstrong and Hearst, 1996) and the enzymes involved in various organisms. Here we discuss how this knowledge may contribute to the understanding of the astaxanthin biosynthesis in *Phaffia* and the regulation thereof. Furthermore the possibilities for the use of recombinant DNA techniques for increased astaxanthin production will be discussed.

Synthesis of geranyl geranyl pyrophosphate

The first step in the synthesis of carotenoids involves the synthesis of geranyl-geranyl pyrophosphate (GGPP, C20) (**FIG. 2**). Starting with acetyl-CoA through 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA), MVA is formed by the highly regulatable enzyme HMG CoA reductase. MVA is converted to the smallest isoprene unit, isopentenyl pyrophosphate (IPP,C5).

IPP may undergo isomerization to dimethylallyl pyrophosphate (DMAPP) by IPP-isomerase (idi). Sequential condensations of DMAPP with IPP's leads to the formation of geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15) and geranylgeranyl pyrophosphate (GGPP, C20).The condensation reactions are catalyzed by one or more prenyl transferases (FPP/GGPP synthetases) depending on the species specific enzyme affinity for one or more prenyl diphosphates. E.g. in yeast, *capsicum* and methano bacterium DMAPP/IPP is converted to GGPP by a single enzyme, whereas in rat and *Phycomyces* more enzymes are involved in this conversion is (Sandmann 1994, for a review).

From especially FPP and GGPP different biosynthetic routes diverge diminishing the flow of substrate to the carotenogenic enzymes that start at GGPP.

Synthesis of β-carotene from GGPP

GGPP serves as a substrate for 6 biosynthetic pathways (Bramley and Mackenzie 1988), including the formation of gibberelins, chlorophylls (Kush 1994) and carotenoids. In the latter GGPP is dimerized to the first colorless carotenoid phytoene (C40) via prephytoene pyrophosphate (PPPP).

Phytoene is subsequently converted into the red intermediate lycopene through four desaturation reactions. This conversion may be catalyzed by one or two desaturases, that belong to three different types, based on the number of desaturation steps that are carried out (Sandmann 1994, for a review). In maize, tomato and *synechococcus* the phytoene desaturase catalyzes two desaturation steps leading to γ-carotene. In *Rhodobacter* three desaturation steps are catalyzed yielding neurosporene as an intermediate. In both cases a second enzyme is needed for the formation of the maximally desaturated

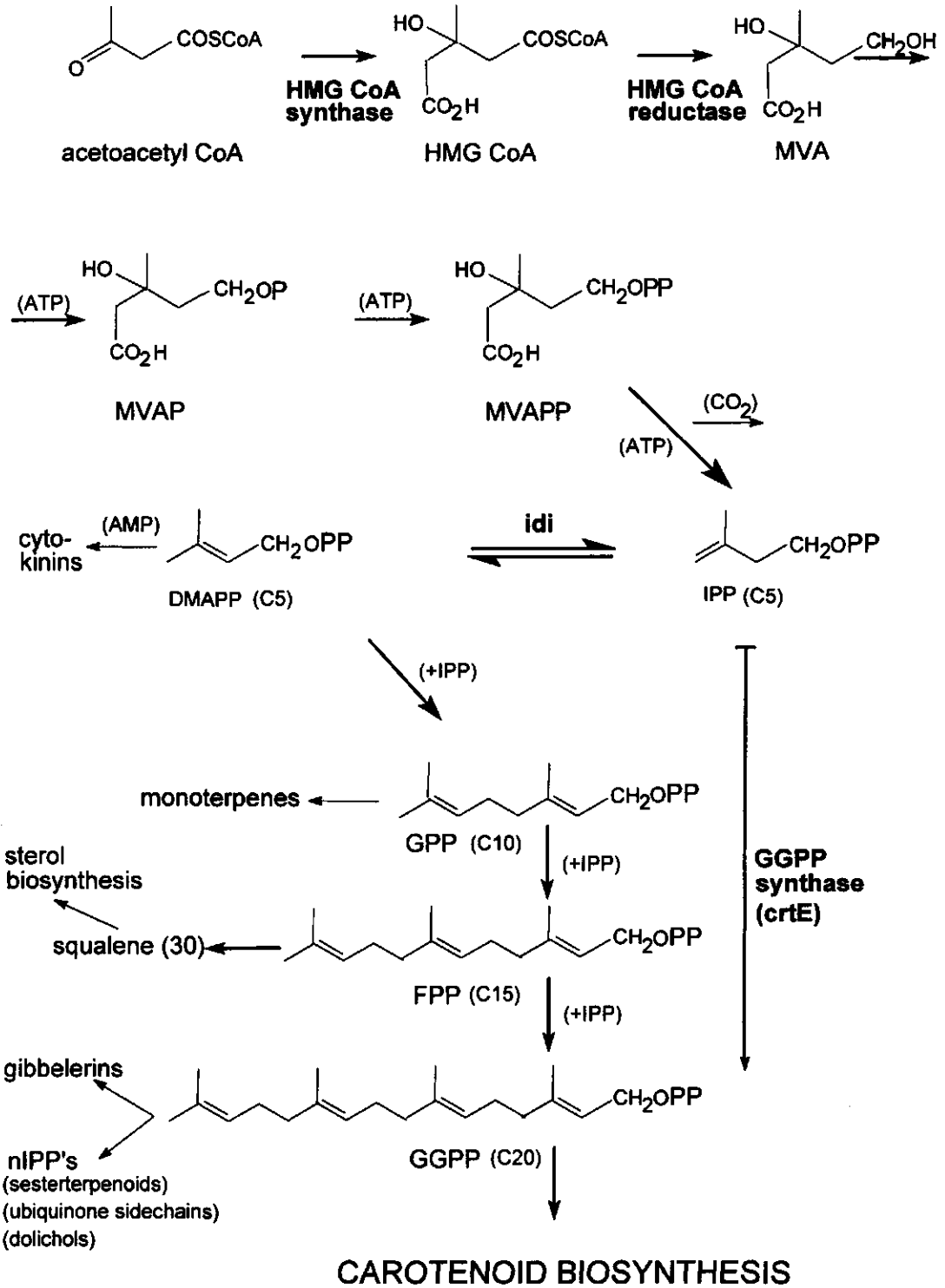
COSCoA
O
HMG CoA synthase
HO
COSCoA
CO2H
HMG CoA reductase
HO
CH2OH
CO2H
acetoacetyl CoA
HMG CoA
MVA
(ATP)
HO
CH2OP
CO2H
MVAP
(ATP)
HO
CH2OPP
CO2H
MVAPP
(CO2)
(ATP)
cyto-kinins
(AMP)
CH2OPP
DMAPP (C5)
idi
CH2OPP
IPP (C5)
(+IPP)
monoterpenes
CH2OPP
GPP (C10)
(+IPP)
sterol biosynthesis
squalene (30)
CH2OPP
FPP (C15)
(+IPP)
GGPP synthase (crtE)
gibbelerins
CH2OPP
nIPP's
(sesterterpenoids)
(ubiquinone sidechains)
(dolichols)
GGPP (C20)
CAROTENOID BIOSYNTHESIS

FIG. 2.

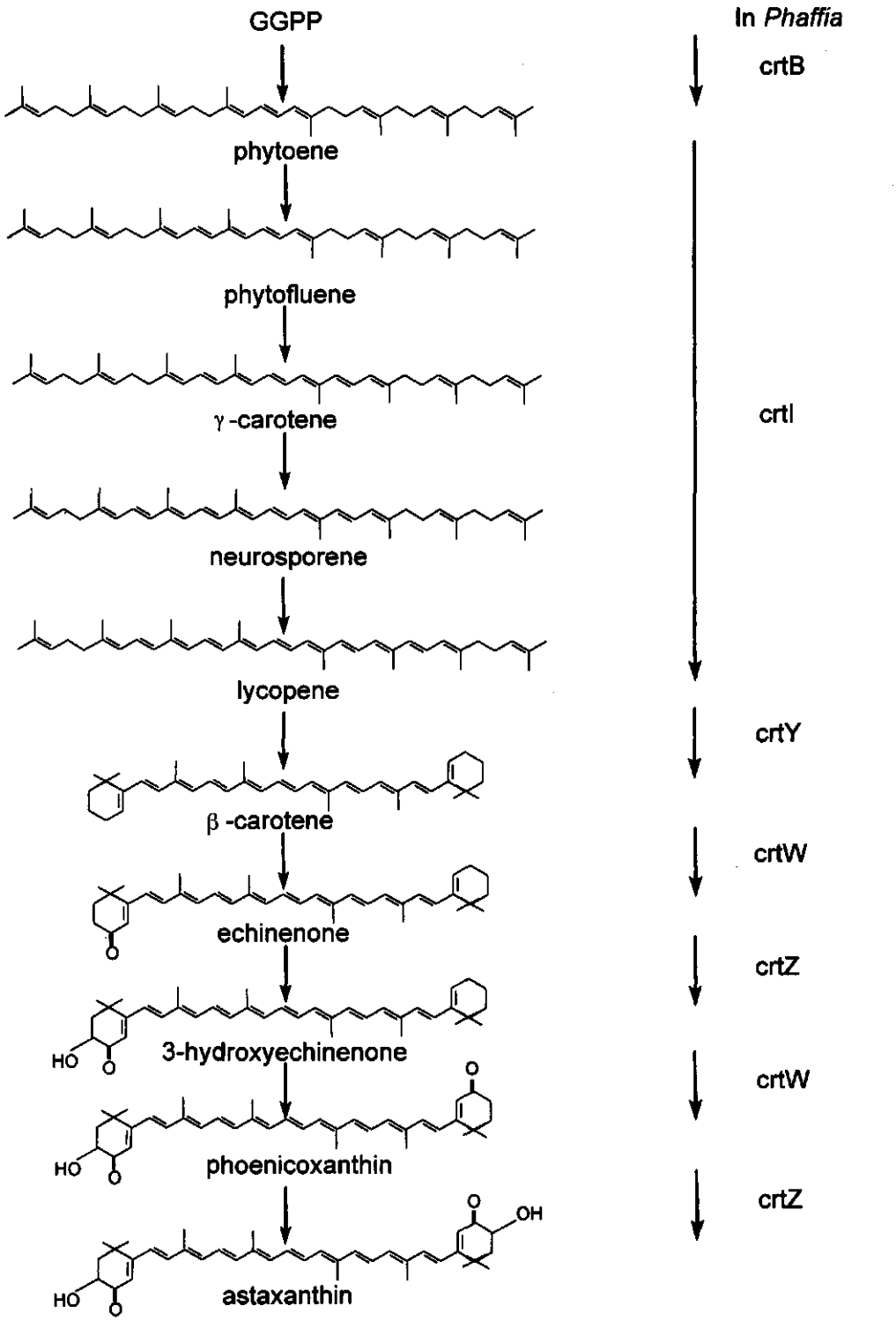

FIG. 2.
General scheme of the biosynthesis of carotenoids

lycopene. In *Erwinia* and *Neurospora* lycopene is formed by one phytoene desaturase catalyzing four desaturation steps.

Lycopene usually serves as a substrate for the formation of β-carotene via γ-carotene by the subsequent formation of two β-ionone rings at either side of the polyene chain. This two step reaction is catalyzed by a single enzyme.

Formation of astaxanthin from β-carotene

Until recently only little was known about the reactions and the enzymology of the formation of xanthophylls, in particular astaxanthin. However, in the past 2 years genes have been isolated from the marine bacteria *A. aurantiacum* (Yokoyama and Miki, 1995; Misawa et al., 1995b), *Alcaligenes* PC-1 (Misawa et al., 1995a), and the green alga *H. pluvialis* (Lotan and Hirschberg, 1995; Kajiwara et al., 1995) involved in the conversion from β-carotene to astaxanthin.

Analysis of the intermediates that are formed by complementation of *E. coli* strains that were genetically engineered to produce β-carotene or zeaxanthin revealed that 2 genes, *crtW* and *crtZ*, are required for the formation of astaxanthin (Misawa et al., 1995b).

It was shown that *crtW*, the *bkt* gene in *H. pluvialis* (Kajiwara et al., 1995), and *crtZ* were responsible for the conversions of methylene to keto and hydroxyl-groups respectively in the β-ionone rings of β-carotene. It was found that both enzymes showed low substrate specificity in that *crtW* converts both β-ionone and 3-hydroxy-β-ionone, where as crtZ converts β-ionone and 4-keto-β-ionone. This allows for the formation of numerous intermediates.

Prediction of the conversions in the carotenoid/xanthophyll synthesis pathway in *Phaffia* is mainly derived from carotenoid composition studies in wildtype and mutants strains either with or without the addition of inhibitors of known carotenogenic conversions.

Carotenoid/xanthophyl synthesis in *Phaffia*

1. *From phytoene to β-carotene*

Girard et al. (1994) have obtained *Phaffia* mutants that accumulate almost all known intermediates in the pathway from phytoene to β-carotene. However, only phytoene and β-carotene could be produced by some mutants as the sole carotenoid in high amounts. Furthermore, the addition of nicotine, which is known to inhibit cyclases, resulted in the accumulation of lycopene as the major carotenoid.

These results suggest that phytoene, lycopene and β-carotene are the final products of enzymatic conversions, implying that in *Phaffia* only two enzymes (one phytoene desaturase and one cyclase) are involved in the synthesis of β-carotene from phytoene. This resembles the situation in *Erwinia* and *Neurospora*.

Recently it was shown that indeed the *crtI* and *crtY* genes of *E. uredovora* could each be functionally complemented by a *Phaffia* cDNA (J. Verdoes pers. comm.).

2. *From β-carotene to astaxanthin*

Contrary to the situation in the astaxanthin producing *A. aurantiacum*, that accumulates 9 different compounds between β-carotene and astaxanthin (Yokoyama and Miki, 1995), in *Phaffia* only 3 intermediates were demonstrated: echinenone, 3-hydroxyechinenone,

phoenicoxanthin (Johnson and Lewis 1979).

Provided that in *Phaffia* also 2 enzymes are involved to convert β-carotene to astaxanthin, their specificity may be higher than the *A. aurantiacum crtW* and *crtZ*.

It would appear that in *Phaffia* the *crtW* analogue only recognizes β-ionone, whilst the *crtZ* analogue has highest specificity for 4-keto-β-ionone. This leads to a strict sequence in the conversions they catalyze. β-carotene would be the sole substrate for the *Phaffia crtW* leading to echinone. The 4-keto-β-ionone ring in echinone would be converted to a 3-hydroxy-4-keto-β-ionone ring by *crtZ* leading to 3-hydroxyechinone. The β-ionone ring in 3-hydroxyechinone would subsequently be converted by *crtW* and *crtZ* in the same order leading to astaxanthin via phoenicoxanthin.

The specificity of the *Phaffia crtW* would also differ from its analogue in *H. pluvialis*, *bkt*, since the latter has been shown to efficiently convert β-carotene to canthaxanthin.

For optimization of the carotenoid production in *Phaffia* with use of recombinant DNA techniques, it is important to know which conversion in the multi-enzyme pathway is rate limiting. Although little is known about the regulation of carotenogenic genes in *Phaffia*, several investigations in other organisms have revealed that especially in the well conserved MVA pathway highly regulated genes exist. Overexpression of these "bottle-neck" genes in *Phaffia* could ultimately improve astaxanthin production.

Bottleneck-conversions in the synthesis of astaxanthin

1. *HMG-CoA reductase*

MVA is a precursor in the biosynthesis pathway of isoprenoids like carotenoids. It was shown by Calo et al. (1995) that during cultivation of *Phaffia* in the presence of 0.1% MVA, astaxanthin production was increased 4 fold.

The synthesis of MVA is highly regulated to both ensure a constant flow of essential compounds and to avoid the overproduction of potentially toxic sterols. It was shown that upon depletion of MVA the expression of the enzyme HMG-CoA reductase, catalyzing the conversion of HMG-CoA to MVA, can be enhanced on the transcriptional, translational and post-translational level yielding a 200 fold increase of the protein within few hours (Brown et al., 1978; Nakanishi et al., 1988). The adverse effect can be obtained by addition of MVA and/or sterols indicating a strong feed back inhibition of the enzyme.

Overexpression of HMG-CoA reductase in carotenoid synthesizing organisms may lead to the shunting of GGPP into the carotenoid biosynthesis pathway to avoid a lethal accumulation of sterol-like compounds.

2. *IPP-isomerase*

The isomerization of IPP to DMAPP by the enzyme idi is a key step in the synthesis of GGPP in yeast, *capsicum* and methano bacterium. It was shown recently by Verdoes et al. (1996) that the presence of the *Phaffia* idi gene on a multi-copy plasmid in an genetically engineered *E. coli* strain, that accumulated lycopene, greatly enhanced the carotenoid production. This result indicated that overexpression of idi results in an optimal DMAPP/IPP ratio to serve as substrates for the synthesis of GGPP. Excess GGPP, that can normally only detected in trace amounts in *E. coli*, is apparently diverted into the carotenoid biosynthesis pathway. It is not clear to which compound (DMAPP or IPP) the reaction is driven by overexpression of *idi*.

3. GGPP synthase

GGPP is found in many organisms, including non-carotenogenic, and serves as a substrate for the synthesis of a variety of (essential) compounds as gibbelerins, sesterterpenoids, ubiquinone side chains and dolichols (Bramley and Mackenzie 1988).

Despite the fact that GGPP is not a carotenoid, two observations led Sandmann (1994) to conclude that the start of the carotenoid biosynthesis starts with GGPP synthase (crtE). First, the carotenogenic gene clusters in all bacteria investigated included *crtE*. Second, it was shown by Kuntz et al. (1992) that overexpression of the GGPP synthase gene in *capsicum* coincided with a massive production of carotenoids. The last observation also indicated that the formation of GGPP may be a rate limiting step in carotenoid production. In fact, it was already found by Brinkhaus and Rilling (1988) that in a β-carotene overproducing strain of *Phycomyces* 4 times higher levels of GGPP synthase levels were present.

4. Phytoene synthetase

The conversion connecting the MVA pathway to the first carotenoid, GGPP ⇒ phytoene, has been shown to be rate limiting in the bacterium *T. thermophilus* (Hoshino et al., 1993). A strain containing the gene encoding for this conversion, *crtB*, on a multicopy plasmid, produced about 3 times more carotenoids than the wt strain.

Carotenoid production could be further optimized to a maximum of 20 times higher by introduction of multiple copies of the gene in carotenoid overproducing mutants of the bacterium.

Strategies to improve production of astaxanthin in *Phaffia* with recombinant DNA technology

From the foregoing it can be deduced that the production of carotenoids can be significantly enhanced with recombinant DNA technology. Two observations support that this is also true for *Phaffia*.

First, multiple copies of the *Phaffia idi* gene resulted in an enhanced carotenoid expression in *E. coli*. The conserved nature of this part of the carotenoid biosynthesis pathway leads us to expect that overexpression of *idi* leads to enhanced carotenoid production in *Phaffia*.

Second, it was found in our lab that introduction of multiple copies of the *Phaffia crtB* gene, using the transformation system described in CHAPTER 5, led to a 2-3 fold higher astaxanthin production per gram dry weight compared to wildtype strains (J. Verdoes pers. comm.). Although the use of classical mutagenesis has yielded *Phaffia* strains that produced up to 10 fold more astaxanthin compared to wildtype, this result is promising as a first step towards development of recombinant strains that carry even more astaxanthin biosynthesis genes in multiple copies.

Based on the current knowledge, optimization of carotenoid production should be focused on the overexpression of 3 additional bottle neck genes in the astaxanthin biosynthesis pathway, HMG CoA-reductase, IPP-isomerase *(idi)* and GGPP synthase *(crtE)*. The overexpression of these genes involved in the early pathway of astaxanthin biosynthesis may urge the cell to divert the increased carbon flow from GGPP into the carotenoid biosynthesis.

Due to its physical properties, like a hydrophobic polyene core and polar headgroups, astaxanthin is likely to interfere with the cell membrane affecting its structure and vital functions. Overexpression of the xanthophyll may therefore have a toxic effect on the cell. Therefore the proper stacking of (overproduced) astaxanthin would be important to avoid possible deleterious effects.

In *Phaffia* astaxanthin was shown to reside mainly in lipid globules near the cytoplasmic membrane. Furthermore, the level of astaxanthin production and the amount of lipid globules appeared to be correlated. Lipid globules contain isoprenoids and like carotenoids, their production is highly dependent on the MVA biosynthetic pathway. Hence, for a maximum yield, the enhanced production of carotenoids at the expense of the formation of other isoprenoids should be avoided. This is an extra argument to not only focus on the introduction of multiple copies of *crt* genes, but also of early pathway genes.

Finally for the successful development of *Phaffia* as a commercially attractive astaxanthin source, the joint application of classical mutagenesis and recombinant DNA techniques would offer best prospects.

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Summary

The aim of this thesis is studying the genetics of *Phaffia* and to develop a genetic transformation system for this yeast. The genetic properties of *Phaffia* were studied on the gene and genome level.

As a first step the molecular structure of the *Phaffia* actin gene was analyzed. Actin genes are highly conserved throughout nature, and as such they have been used for the classification of significantly diverging eukaryotic groups, like (in-)vertebrates, plants and fungi.

We anticipated that the analysis of the primary structure of *Phaffia* actin gene and comparison with the actin genes from fungi, including 2 ascomycetous filamentous fungi, 2 basidiomycetous yeasts and 5 ascomycetous yeasts would provide further phylogenetic information on this yeast.

It was found that the *Phaffia* actin gene encoded a protein consisting of 375 amino acids. In addition 4 (non-coding) intervening sequences were present. Comparison of both the coding DNA sequence and its predicted protein product with their fungal counterparts, revealed that least homology was found with the ascomycetous yeasts, like *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. It was also shown, that based on these comparisons *Phaffia* is closer related to the filamentous ascomycetous fungi *Thermomyces lanuginosus* and *Aspergillus nidulans*, whereas most homology was found with the basidiomycetous yeast *Filobasidiella neoformans* (perfect stage of *Cryptococcus neoformans*).

In addition to the phylogenetic analysis of the actin exons, the architecture of the introns (splice site consensus sequences, size, position in the gene) was compared. It was shown that the *Phaffia* introns most resembled that of *Filobasidiella neoformans*, where as least resemblance occurred with the ascomycetous yeasts. This result was in agreement with the actin exon homology studies. Furthermore, the presence of multiple introns in the *Phaffia* actin gene resembled the situation in the actin genes from *F. neoformans* and the filamentous fungi, whereas the ascomycetous yeasts only carry one intron in their actin genes.

Similar results were obtained by (phylo-)genetic analysis of the five introns containing *Phaffia* glyceraldehyde-3-phosphate dehydrogenase gene.

The genomic organization of the multiple rDNA genes in *Phaffia* was elucidated. It was found that *Phaffia* carries the rDNA genes in three clusters, of 12, 14 and 35 copies, on three different chromosomes. In the ascomycetous yeasts and fungi the rDNA is mainly present on one chromosome.

The significant differences on the gene and genome level with the ascomycetous yeasts affected the strategy for the development of a transformation system for *Phaffia*. Whereas several marker gene sequences or sequences for plasmid replication and maintenance can be readily interchanged between most ascomycetous species as a result of high homologies, it was shown that this was not the case for *Phaffia*. Therefore an almost entirely homologous transformation sytem was developed using plasmids carrying the dominant G418 resistance gene (Km^R), driven by either the *Phaffia* actin or the *gpd* promoter and a *Phaffia* ribosomal DNA (rDNA) fragment for homologous integration.

It was found that the rDNA clusters could serve as a target for high copy number integration. This integrative transformation system was used to determine the ploidy of *Phaffia*, strain CBS 6938, by monitoiring chromosomal shifts as a result of multiple integrations. It was found that this strain was haploid.

Plasmids carrying the *gpd* promoter driven Km^R gene transformed *Phaffia* with signi-

Summary

ficant higher efficiencies than constructs with the actin promoter. Furthermore, the plas-mid copy number and transformation efficiencies of the first were found to be influenced by the presence of the *gpd* terminator downstream the Km^R gene.

It was shown that plasmid amplification occurred independent from selection pressure to an extend that appeared to be negatively related to the effectiveness of expression of the Km^R gene. This observation indicated that the rising metabolic burden, as a consequence of amplification, imposes limits to the number of plasmid copies.

The effectiveness, stability, and plasmid amplifying properties of the *Phaffia* transformation system offer possibilities for the use of recombinant DNA technology in developing industrially attractive *Phaffia* strains.

Samenvatting

De doelen van het onderzoek zoals beschreven in dit proefschrift, waren bestudering van de genetica van de gist *Phaffia rhodozyma*, zowel op het gen- als genoomniveau, en de ontwikkeling van een genetisch transformatie systeem.

Op het genniveau werd de structuur van het actine gen van *Phaffia* bestudeerd. Actine genen zijn sterk geconserveerd in eukaryoten en zijn, vanwege die eigenschap, eerder gebruikt voor de classificatie van uiteenlopende phylogenetische groepen, waaronder (on-)gewervelden, planten en schimmels. Om een beter inzicht te krijgen in de plaats van *Phaffia* binnen de verschillende taxonomische schimmel en gist groepen, werd de primaire structuur van zijn actine gen geanalyseerd en vergeleken met actine genen van uiteenlopende gisten en schimmels. Hieronder waren 2 ascomycetische filamenteuze schimmels, 2 basidiomycetische- en 5 ascomycetische gisten.

Het *Phaffia* actine gen bleek te coderen voor een eiwit van 375 aminozuren en er bleken 4 intronen aanwezig. Uit de vergelijking van zowel de nucleotide volgorde van de exons en de aminozuur volgorde van het voorspelde eiwit, bleek dat het *Phaffia* gen het minst gerelateerd was aan die van de ascomycetische gisten, zoals *Saccharomyces cerevisiae* en *Kluyveromyces lactis*. De homologie met de actine genen van de filamenteuze ascomycetische schimmels *Thermomyces lanuginosus* en *Aspergillus nidulans* en vooral van de basidiomycetische gist *Filobasidiella neoformans* (perfecte stadium van *Cryptococcus neoformans*) bleek groter.

Verder werd de architectuur (aantal introns, consensus nucleotide volgorde van de splice sites, grootte, lokatie in het gen) van de actine introns vergeleken. Ook hier bleek het actine gen van *Phaffia* het meest verwant met dat van *Filobasidiella neoformans* en het minst met die van de ascomycetische gisten.

Vergelijkbare resultaten werden verkregen uit de (phylo-)genetische analyse van het glycolytische *Phaffia* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gen.

Op het genoomniveau werd de organisatie van de meervoudige ribosomaal DNA (rDNA) genen in *Phaffia* bestudeerd. Deze genen bleken voor te komen in 3 clusters van 12, 14 en 35 kopieën verdeeld over 3 chromosomen. In ascomyceten zijn de rDNA genen voornamelijk op 1 chromosoom aanwezig.

De verschillen tussen *Phaffia* en de genetisch bekendere ascomycetische gisten, op zowel gen als genoom niveau, waren van invloed op de ontwikkeling van een transformatie systeem. Het bleek dat marker genen en sequenties voor plasmide replicatie, die gebruikt worden voor de ascomycetische gisten, niet voor *Phaffia* geschikt waren. Er werd derhalve een bijna geheel homoloog transformatie systeem ontwikkeld, bestaande uit plasmiden met als marker het dominante G418 resistentie gen (Km^R), aangedreven door de *Phaffia* actine- of *gpd* promotor, en met een *Phaffia* rDNA fragment voor homologe integratie.

Deze plasmiden bleken in hoog aantal (20-60 kopieën) te integreren in de rDNA clusters van het *Phaffia* genoom. Dit integratieve transformatie systeem kon zo onder andere gebruikt worden om de ploidy van *Phaffia* CBS6938 te bepalen, door het chromosomale banden patroon van transformanten te bestuderen en te vergelijken met die van het wildtype. *Phaffia* bleek haploid.

Plasmiden met het Km^R gen aangedreven door de *gpd* promotor werden effectiever naar *Phaffia* getransformeerd dan plasmiden met de actine promotor. Bovendien bleek de aanwezigheid van de *gpd* terminator achter het Km^R gen de transformatie efficiëntie nog aanmerkelijk te verhogen.

Samenvatting

De vermeerdering van het aantal plasmide-kopieën in *Phaffia* transformanten leek onafhankelijk van selectie druk. Het uiteindelijke aantal plasmide-kopieën bleek negatief beïnvloed te worden door de aanwezigheid van de *gpd* terminator achter het Km^R gen. Hieruit zou afgeleid kunnen worden dat de toenemende metabole belasting als ge-volg van plasmide vermeerdering grenzen stelt aan het uiteindelijke aantal plasmide kopieën.

De effectiviteit en de stabiliteit van het *Phaffia* transformatie systeem en het grote aantal plasmide-kopieën openen mogelijkheden voor het vervaardigen van industrieel aantrekkelijke *Phaffia* stammen.

Nawoord

Gedwongen na te denken over dit nawoord, besef ik dat dit proefschrift er zonder de direkte en indirekte steun van een aantal mensen niet tot stand was gekomen. Ik neem deze gelegenheid dan ook graag om deze mensen te bedanken, zij het voor een aantal wat laat.

Ab van Ooyen, mijn promotor, ik wil je hartelijk bedanken voor je steun, je vertrouwen, je meezweten, en je relativerende kijk op de zaken. Je verhalen over vroeger, over de bijna niet te nemen obstakels in jouw promotie onderzoek, en de harde tijd in Zwitserland, waren altijd een steuntje in de rug in tijden dat mijn eigen onderzoek bepaald niet vlotte. Jan Verdoes, mijn collega en vriend, bedankt voor je inzet en meedenken. Het was geen toeval dat mijn onderzoek pas echt ging lopen toen jij bij onze club kwam. Mieke Dalderup, Ton Renniers en Diana Gutker, mijn collega's bij Gist-brocades, jullie werk vormde een stevig fundament om mijn onderzoek op voort te bouwen. "Mijn" afstudeerders, Harald Ruijssenaars, Woutera Bakker, José ter Linde, Frank Brouwers, Lucien Montulet en Roelie Kingma, bedankt voor jullie grote inzet en enthousiasme. Al mijn ex- en toekomstige collega's van de sectie Industriële Microbiologie, bedankt voor de goeie werksfeer en collegialiteit. De kroegtochten met in het bijzonder Harald Ruijssenaars, Hermann Heipieper, Sonja Isken, Gert Grobben en Jelto Swaving en het oeverloze gezwam over niks hebben me goed gedaan.

Ru en Jopie ik wil jullie bedanken voor je interesse en steun "achter de schermen".

Pa en Ma, aan jullie heb ik zoveel te danken, maar vooral het besef dat het leven uit meer bestaat dan een promotie-onderzoek. Mijn lieve Toefje, steun en toeverlaat onder alle omstandigheden, bedankt dat je altijd en onvoorwaardelijk naast me stond.

Tot slot wil ik dit proefschrift opdragen aan mijn helaas overleden dierbare grootvader en voorbeeld, Jan Wassink en aan mijn grootmoeder Jacoba Wassink.

Curriculum vitae

Jan Wery werd geboren op 8 augustus 1966 te Utrecht. Hij haalde het VWO diploma in 1984 aan het Eemland College Zuid te Amersfoort en vervulde vervolgens gedurende 16 maanden zijn dienstplicht als sergeant bij de Koninklijke Landmacht. In 1986 begon hij zijn studie Biologie, oriëntatie Cel, aan de Landbouwuniversiteit Wageningen. Deze studie werd in 1992 afgerond met de 6 maands afstudeervakken Moleculaire Biologie en Industriële Microbiologie en een stage van een half jaar bij The Department of Molecular Genetics and Microbiology, aan de State University of New York at Stony Brook in de Verenigde Staten. Van 1992 tot 1996 was hij Assistent in Opleiding bij de sectie Industriële Microbiologie van de Landbouwuniversiteit Wageningen, en verrichtte hij het onderzoek dat beschreven is in dit proefschrift. Van mei tot augustus 1997 deed hij als gastmedewerker onderzoek bij het Center for Agricultural Molecular Biology van de Rutgers State University of New Jersey in de Verenigde Staten. Sinds 1 september 1997 is hij werkzaam als wetenschappelijk onderzoeker aan de sectie Industriële Microbiologie van de Landbouwuniversiteit Wageningen.

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