TARGETED ADAPTATION

DEVELOPMENT OF A CROSSOVER-SPECIFIC PCR ASSAY ANALYZING THE ASSOCIATION BETWEEN TRANSCRIPTION AND MEIOTIC RECOMBINATION

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Targeted Adaptation: Development of a Crossover-Specific PCR Assay Analyzing the association between Transcription and Meiotic Recombination

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Abstract

Genetic recombination is the characterizing trait of sexual reproduction, with crossovers between homologous chromosomes being necessary for correct segregation of chromosomes in meiotic division. However, recombination can be advantageous or disadvantageous to the progeny depending on the type of epistatic interactions broken in the process. In this thesis, I hypothesize that transcription induces crossover formation in the meiotic division of plants and fungi. High transcription of genes would identify loci where recombination would be most favourable for adaptation and survival in local environments. This can explain the variability of crossover hotspot positions in different environments, localization at transcription start sites, the epigenetic elements associated with hotspot positions and the strong association between the evolution rate of genes and transcription. To measure the recombination frequency within a region spanning a few kilobase pairs, I started the development of a previously described crossover-specific PCR assay in *Arabidopsis thaliana* cytoswap strains. Furthermore, I attempted to develop the technique in *Aspergillus fumigatus,* an organism that tolerates fungicide stress by inducing high levels of transcription of the target gene Cyp51A. The loci chosen for the development of the protocol proved to be unsuitable for the assay in both species. Crossover- specific PCRs require a large population of meiotic progeny, and polymorphisms enabling the design of allele-specific primers. I further discuss recommendations for the choice of genes and treatments for future trials and alternative approaches to measure the relationship between transcription and crossover formation. If crossover formation is indeed influenced by transcription, this has big implications for evolution in sexual organisms, as well as for plant and fungus breeding for biotic and abiotic stress resistance.

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Introduction

Unless you work in a microbiology lab, the chances are that most of the life you see has been generated through sexual reproduction. In the fundamental process of meiotic division, a cell recombines DNA from two parents, present in separate molecules, and faithfully produces four gametes. The cycle is closed with the fusion of two compatible gametes to produce a new diploid capable of repeating the process. Meiosis is widely considered to originate alongside eukaryotic organisms, and is necessary for the long-term survival of individual species in this domain of life[1]. Humanity has used this mechanism over thousands of years to select the most useful individuals of plant and animal species, leading to domestication[2]. As such, it is fundamental in our understanding of natural selection, with only fit organisms contributing genetic material to the next generation.

A simple definition of fitness is the amount of progeny an individual produces in its lifetime[3]. As with many phenotypic traits, this is a product of the genetic composition of an individual and the environment it is found in. On the genetic side, this is an extremely, if not the ultimate, complex trait, requiring the presence and balance of many different coded elements involved in growth. Furthermore, systems to perceive external stimuli give specific organisms an advantage over others to benefit from opportunities and respond to threats in changing environments[4].

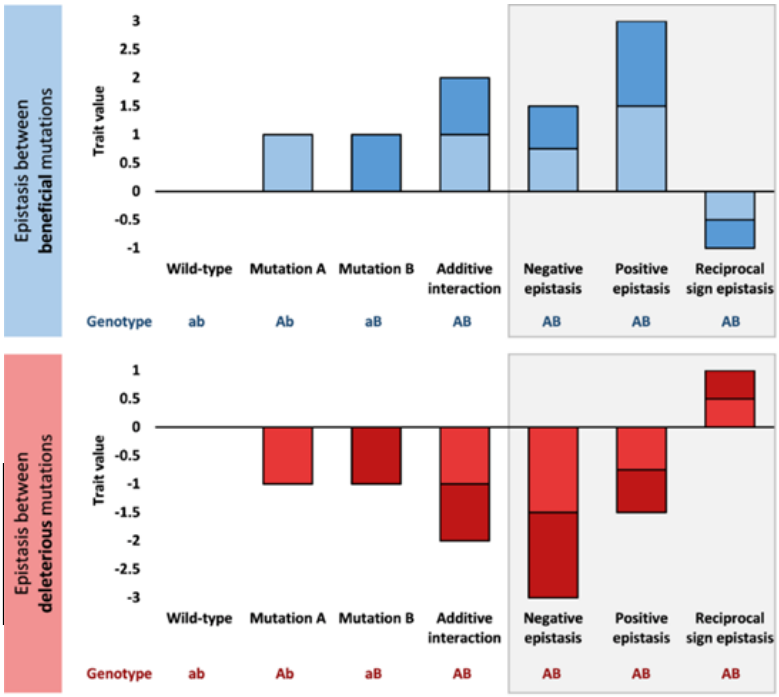
The progeny of an organism will most likely find itself in the environment inhabited by its parents [5]. Given a theoretical environment, a clone will conserve the fitness of its parent, and over time, the fittest phenotype will dominate the niche. The advantages of asexual reproduction, widespread among the prokaryotic world, has beggared scientists for generations as to the benefits of sexual reproduction. Scholars have pointed out the many costs of sex, not least the very recombination that is the hallmark of the process. Recombination admittedly can break off deleterious mutations and facilitate the accumulation of beneficial alleles. But it can also separate beneficial genes that work in combination for survival and environment response. In this thesis, I shall argue that recombination is preferentially located to specific regions to maximize the benefits and minimize the costs of sex.

1.1 Redundancy, complexity and gene interactions

In order to understand the costs and benefits of recombination, it is important to understand genetic interactions. Epistasis is the observable phenomenon of two gene alleles presenting a different effect together than what is expected from their individual effect[6]. The alternative is an additive effect, where the phenotypic contribution of two genes are independent from each other.

Epistatic effects can span a wide spectrum of interaction, from dominant epistasis, where the phenotypic contribution of one allele completely overshadows the contribution made by other alleles, down to minor

differences from the expected additive effect[7]. These phenomena can be further classified into positive effects, leading to a benefit in the presence of two alleles, negative epistatic effects, where the phenotype is worse than expected, and sign interactions that show the reverse of the expected phenotype (Fig.1).



Epistasis may be a necessary phenomenon to develop genome complexity. Redundancy, between both genes and entire metabolic pathways, protects against random deleterious mutations. This allows the otherwise impossible development of new alleles via non-fatal intermediate steps[8]. However, this can produce negative epistasis, as interchangeable pathways compete to produce a phenotype rather than contribute efficiently to the trait in an additive manner[6]. On the other hand, genes requiring the presence of compatible alleles at other loci produce phenomena of positive epistasis. The association between complexity and epistasis has been made further manifest by empirical reviews [9]and in-silico studies[10]. In these studies, the complexity of genomes, defined as larger in size, gene count and robust, correlate with the effect of epistasis on quantitative traits.

There is evermore documentation on epistasis, in large part thanks to the improvement of analytical capabilities to detect these interactions[7]. An example of this is the high throughput gene interaction profiles identified for *Saccharomyces cerevisiae*[11]. In this study, 18 million interactions between ~90% of yeast genes were reviewed for their effect on growth. Approximately 900.000 were non-additive, with two-thirds of the interactions being negative and a third positive. This highlights the large contribution of gene interactions to the growth trait, and the predominance of negative over positive interactions in *S. cerevisiae*. Noteworthy of mention, the environment has an impact on epistatic interactions, exemplified by research into the glucosynolate hormone synthesis pathway in *Arabidopsis thaliana* under different degrees of stress [12].

1.2 The consequences of recombination: breaking up gene interactions

As discussed previously, complex genomes contain a multitude of interactions between genes. These remain largely conserved in asexual reproduction, where the genome is transferred intact from parent to progeny. This characteristic allows for safe proliferation in environments where allele combinations are essential for survival. Cloning can also be an effective strategy for rapid proliferation under favourable conditions due to the relatively low cost of asexual reproduction [13]. Both of these phenomena have been observed in plants that present both sexual and asexual reproduction[14]. Clonally reproducing organisms diverge over time via mutation, generating the Meselson effect, where asexual organisms present greater allele divergence than sexual organisms[13]. Simple organisms can select the individuals best adapted to the environment from large populations[8]. Complex organisms however may depend on the integrity of specific gene interaction networks. If deleterious mutations

occur linked to beneficial alleles, this can lead to the Muller's ratchet effect, reducing the fitness and eventually extinguishing the species[15].

By definition, recombination during sexual reproduction leads to the exchange of genetic material between two haploid genomes. This has the benefit of restoring damaged networks and creating new allele combinations, accelerating the adaptation of the population to the environment. However, recombination can also have a detrimental effect, breaking up positive combinations of alleles and replacing them with worse substitutes[15]. This can be especially true in genes, where epistatic interactions are often very strong[16].

To address these concerns, academics have turned to computation to study the effects of epistasis. In one study, modelling computations involving linked, epistatic loci under driving selection were performed. Over the course of generations, a third independently segregating locus favouring recombination is fixed over time in the presence of weak negative and fluctuating epistasis only. Positive and additive loci benefit greatly from recombination in early randomised stages, which becomes limiting once the population accumulates the best alleles in the same individuals[17]. Potential alternative recombination modulating factors are not discussed in the article, however could produce similar evolutionary consequences. A further study highlights the preferential localization of recombination in regions of low epistasis located between regions of high epistasis, mimicking highly epistatic genes and intergenic regions[16].

The eukaryotic genome structure of linear chromosomes is well structured to provide different recombination environments for interacting genes. By separating DNA into independently segregating chromosomes, interactions favoured by recombinations can be positioned on different molecules or on extreme ends of the chromosomes. Epistatic loci benefiting from direct inheritance can be positioned on the same chromosome, requiring a meiotic crossover event between them for recombination to occur. There is much study into the evolutionary clustering of positively interacting metabolic gene clusters[18][19][20] and tissue- specific genes[21] in many species, highlighting this clustering of positively interacting genes over time. Furthermore, genome structure evolution is non-random and dependent on the type of environment[22][23], modulating the amount and location of recombination in the genome.

1.3 Recombination: a far from random process

Meiotic crossovers, and by association intrachromosomal recombination, are the characteristic part of meiotic division. In most species, it is a necessary part of meiosis, ensuring the correct segregation of genetic material between the progeny and avoiding aneuploidies. Despite this, crossover events are generally limited to between one and four per chromosome[24]. Due to this limit, the position of crossover events acquires great relevance, as the number of new gene associations, and thus new epistatic effects, is determined by their position[25]. Importantly, crossover events are spaced out non-randomly between themselves across the chromosome, increasing the recombinational impact of crossovers[26].

Recombination events begin with a double strand break (DSB) in the DNA, which in most eukaryotes is catalysed during meiosis by the conserved protein SPO11[27]. Around 200 DSBs are produced across the wild-type *S. cerevisiae* genome per meiosis[28]. These DSB's are then repaired via homologous recombination. As such, they are often used as a predictor of recombination positions. Studies of the distribution of DSBs can be performed using Chromosome ImmunoPrecipitation (ChIP) sequencing of 20-100bp fragments bound to SPO11 after endonuclease digestion. In *S. cerevisiae*, these trials indicate DSB's are suppressed within 10kb of centromeres and telomeres. Furthermore, 88.2% of DSB hotspots in the same study (defined as 2.3 times average genome

DSB rate) were located in promoter regions[29]. Similar studies have been performed in other organisms, highlighting the non-random nature of DSBs[3031].

However, DSB's do not always produce crossover events. During meiosis, the non-sister chromatid is used as a repair template, and depending on the resolution of the repair-intermediate, DSB repair can either generate a meiotic crossover, or not (i.e. a non-crossover)[32]. Non-crossover events can lead to the non-reciprocal exchange of sequence information close to the DSB site, and the generation of a gene-conversion event, in which case SNPs will segregate in a 3:1 ratio. Crossover events lead to the reciprocal exchange of chromatid arms between non-sister chromatids, often accompanied by associated gene-conversions close to the DSB site. Interestingly, the number of crossovers does not increase proportionally to the number of DSB's, but rather in a logarithmic fashion, limiting the amount of recombination on the chromosome. This phenomenon is known as crossover homeostasis[26].

Another way to study crossover distribution is sequencing the whole genome of meiotic progeny. By observing the variation in SNPs, the position where a crossover has occurred can be estimated, alongside gene-conversion tracts in polymorphic loci [32]. These studies have been performed in *Arabidopsis thaliana* as well as *S. cerevisiae.* and other species. In *A thaliana,* crossover events occur primarily at transcription start sites (TSS) and transcription termination sites (TTS) of loci presenting low DNA methylation (Fig. 2). Crossovers are underrepresented within gene bodies, and are associated with specific transcription factor binding sites[32]. This data was corroborated in a later study, with further associations with histone types associated with transcription such as H3K4me3 and H2A.Z [33].

However, not all organisms adhere to the promoter hotspot consensus. In humans and mice, recombination positions are largely determined by the histone methyltransferase PRDM9, which binds to specific intergenic DNA sequences and recruits SPO11 to the region[34] (Fig.2). PRDM9 histone methylase generates H3K4me3, reminiscent of the actively transcribed genes mentioned previously. This association is confirmed in PRDM9 knockout mice, which revert crossover hotspots to H3k4me3 containing promoter regions[35].

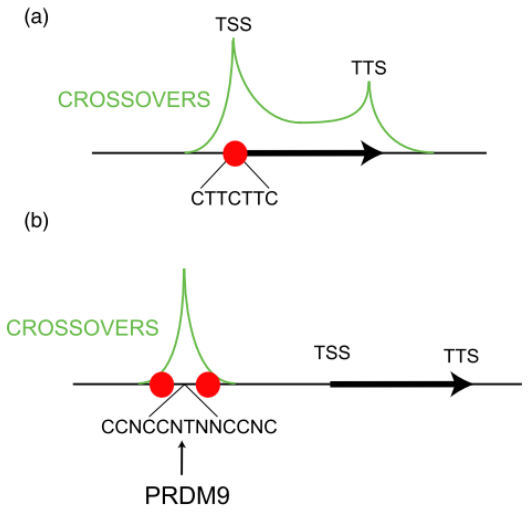


Fig.2: Plant (a) vs human (b) crossover hotspot localization.

In plants, crossover hotspots are primarily located at transcription start sites (TSS) containing CTT-repeat DNA motifs, with a slight peak at transcription termination sites (TTS).

In humans, recombination occurs at intergenic CCN motifs binding to PRDM9, which catalyzes H3K4me3.

Image obtained from Choi & Henderson 2015[30]

1.4 Crossover hotspots are repositioned in different environments

So, how stable are crossover landscapes across the genome in different environments? If we refer to the literature, not very much. All of the crossover landscapes described previously were determined in a standard environment, and many studies have been performed knocking out specific genes involved in meiosis and methylation[30]. On the other hand, studies of the DSB and crossover landscape under different environmental conditions have been less defined.

One of the earliest recognised intraspecies crossover variation was sexual divergence, in large part due to the absence of meiotic crossovers in male *Drosophila melanogaster* meiosis [36]. However, there are also differences in the meiotic recombination landscape in *A. thaliana* gametes[37] and mammal gametes[38]. Age has also been observed to be a factor influencing recombination in *A. thaliana* and *D. melanogaster*[38].

However, not all the factors modulating recombination are intrinsic to the organism. The most well-studied extrinsic factor studied is temperature, as most scientists agree this can directly affect meiotic cells. As such, *A. thaliana* and other plants such as *Hordeum vulgare* present varying recombination landscapes and crossover counts under higher and lower temperatures[38]. An extensive analysis of the DSB distribution of *S. cerevisiae* meiosis at 14°C, 30°C and 37°C showed that only 20% of DSB hotspots are conserved between the three different temperatures[39]. Other extrinsic factors may also play a role, with experimental indications that nutrient composition, liquid vs solid medium and salt concentration affect *S. cerevisiae* crossover placement[40].

A further factor in crossover distribution of plants and yeast may be the degree of heterozygosity between the parents [4041]. With all these factors in mind it appears that the concept of recombination hotspots only applies to specific organisms in specific habitats. Several hypotheses have been put forward to explain this modularity, including physical alterations under temperature[38], specific modulating enzymes and transcription[30]. As of yet, no explanation has been given as to how the progeny of an organism could benefit from modulating recombination patterns.

1.5 Transcription: a potential modulator of recombination

We have seen previously that the value of recombination is dependent on epistasis, and that recombination hotspots are located primarily near TSS of active genes. What is the relationship between these two phenomena? Mitotic homologous recombination has been well associated with transcription[42]. Previous authors have already observed that transcription may be an inducer of meiotic crossovers[30], however, authors are reluctant to make this association due to experimental data showing no effect of transcription on meiotic crossovers [4344]. However, these experiments studied the effects on one position, without considering the global recombination landscape of the genome.

Transcription of a gene into RNA is generally necessary for it to have an impact on the phenotype[45], and as such, is fundamental for intergenic epistasis. Interestingly, genes that have a large number of epistatic interactions tend to have more stable transcription rates across environments[46], possibly to maintain stable degrees of interaction with other genes. Furthermore, many environment response genes are regulated via a feedback mechanism, whereby the presence of stress activates transcription (Fig. 3). This transcription is later inactivated when the stress is no longer

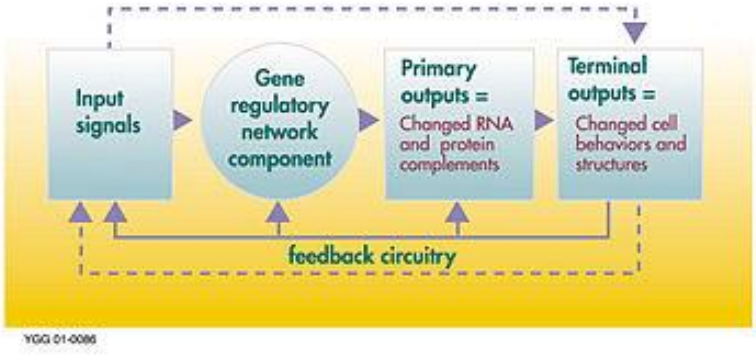


Fig.3: Diagram of a feedback transcription regulation mechanism. Input signals activate gene regulation networks, which induce transcription of RNA and translation of proteins. The terminal output influences (and is influenced) by input signals, regulating further transcription. Image obtained from https://en.wikipedia.org/wiki/Gene_regulatory_network

present[474849]. If a gene is inefficient at responding to a stressor, the transcription of that gene will increase, indicating an environmental limitation and a good locus to increase the speed of adaptation.

Transcription has an energetic cost[50]. As such, it is beneficial for the organism if transcription is limited to the amount of RNA needed for the cell (or organism) to survive. 5,9% of A. thaliana and 3,5% of S. cerevisiae genes are transcription factors[48], directly controlling the amount of transcription in the genome to adapt to environment cues. The other factor regulating transcription rate is the promoter region, which if mutated, can produce diseases in humans[51] and activate defence response genes in plants. When constitutively overexpressed in plants however, disease resistance genes become detrimental to the organisms[52].

Higher transcription rates are associated to higher mutation rates, largely explained by the exposure of single stranded DNA during the process[53]. The immediate consequences of this would be a higher allele diversity in the progeny. Under high transcription rate, the mutation rate of a gene can increase up to 14 times.[54] Despite this, conserved genes are characterized by high transcription and nothing else[55]. This includes related factors such as the codon adaptation index, a measure of how the codons making up a gene match up to the proportion of tRNA present in the cytosol[56]. These articles argue that high transcription increases the selection pressure for efficiency in these genes, however this does not address the fact that highly transcribed genes in *S. cerevisiae* are conserved regardless of whether they are essential or not[57].

1.6 Aim of the thesis

The argument for meiotic recombination targeting transcriptionally active regions of the genome is rather strong, addressing many characteristics present in recombination hotspots. High rates of homologous recombination in highly transcribed genes would explain the conserved nature of highly expressed genes. Breaking up genetic linkage would allow the purging undesirable mutations while ensuring the best alleles for a particular environment are quickly distributed among the population. And finally, it would be a good explanation for the variation of recombination landscapes in different environments.

In this thesis, we shall develop a PCR protocol to measure crossover rate at the gene level. With this technique, we will be able to quantify the variation of recombination rate under different conditions, most notably transcription rate, however other factors such as the influence of transcription factors will also be measurable. We shall attempt to develop the technique in novel organisms, such as cytoswap *A. thaliana* and *Aspergillus fumigatus* strains.

1.7 Crossover-specific PCR

Due to the limited number of crossover events across the genome, crossover events in specific gene locations are predicted to be infrequent events. As such, large sample sizes of progeny in the order of thousands are deemed necessary to detect recombination events in these regions, and further multiples are required to quantify significant differences in frequencies. Crossover- specific PCRs were first developed to detect recombination hotspots from human sperm[58]. This technique has since been adapted to *A. thaliana* pollen for many applications, including determination of crossover frequency variation in DNA methylase mutants. The technique used in this thesis is based on the A. thaliana version described in Choi *et al.*[59].

In this technique, allele-specific primers are designed to bind specifically to the DNA of one parent at a polymorphic site. Using a pair of primers specific to different parents, only molecules that have undergone a crossover event in the region will be amplified. As such, rare recombinant molecules can be detected amongst a pool of non-recombinant DNA. If amplified efficiently, molecules can be quantified using either titration or qPCR techniques (Fig.4).

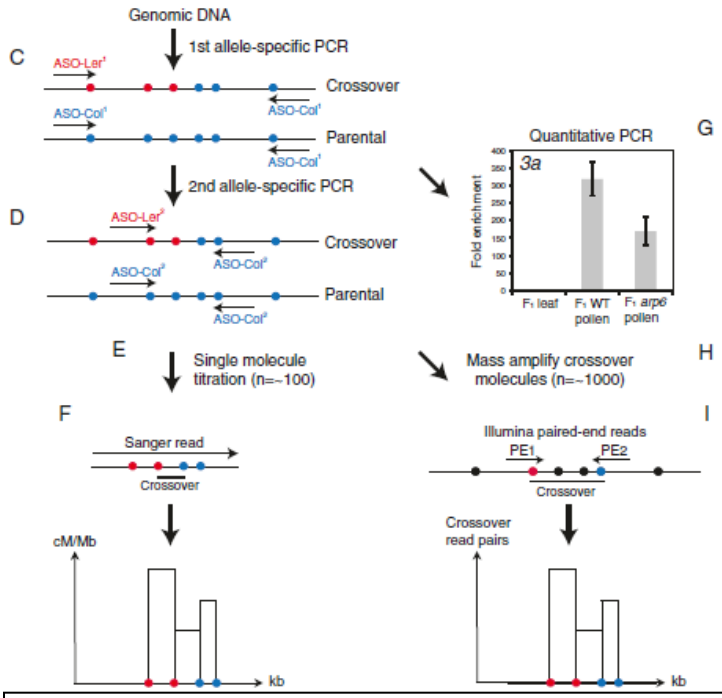


Fig.4: Diagram of an allele-specific crossover. In it, a nested PCR is performed (C & D) with allele- specific primers (marked in red and blue) in order to amplify crossover events specifically. These can then be quantified using, qPCR (G), or titration (E), with sequencing to confirm specific amplification (F&I). Image adapted from Choi *et al.*[58]

The nature of individual allele-specific primers however is unspecific. Due to primers sharing a large proportion of the sequence between strains, they can often anneal to the alternative allele at low frequencies. This is exacerbated at lower temperatures, making primer design process laborious. Choi et al. use in their technique universal primers that can anneal efficiently at high temperatures. Allele-specific primers can then be tested at temperature gradients and selected for specific amplification of the region. To ensure the specificity of the measure amplification, a nested PCR is conducted with a further set of allele-specific primers found within the first amplicon.

1.8 Cytoswap *Arabidopsis thaliana*: measurable differences in photosynthesis

Arabidopsis thaliana is the choice plant species for genetic analysis. As such, it is often used for research in the basic functionality of plant genetics before application on more useful crops. Recently, there has been much effort to explore ways of increasing photosynthetic efficiency in plants as a way to address the increasing demand for agricultural produce[60].

One of the factors observed to influence photosynthetic efficiency is the psbA gene located in the chloroplast genome. In order to study the interaction between the nuclear and cytosolic genomes, a set of *A. thaliana* strains have been developed using the so-called cybrid technique[61]. Unpublished information from this study reveals that there are expression effects in genome transcription in the presence of chloroplast genomes from different *A. thaliana* strains (Theeuwen *et al.,* unpublished).

Since this cytoplasmic effect is present in meiotic cells of *A. thaliana,* it is technically feasible to create an F1 with different cytoplasmic DNA, inducing different expression profiles in otherwise equivalent nuclear

genomes. Using the method introduced above, primers can be designed to quantify the recombination under the influence of different cytosolic genomes.

1.9 *Aspergillus fumigatus*: rapid adaptation to stressful environments

Aspergillus fumigatus is a saprophytic fungus found in all kinds of environments, named for the characteristic cloud of asexual spores that is released on contact. Since the advent of immunosuppressant treatments and antibiotics, *A. fumigatus* has been observed to colonise the respiratory tract of vulnerable patients, causing death if left untreated. For many years, aspergillosis has been treated with azole fungicides, which remain the only effective treatment for the disease. However, in recent years, the number of resistant strains has increased rapidly, presenting a huge medical challenge due to the lack of alternative treatments to the disease[62].

The major target of azole compounds is the enzyme Cyp51A, an enzyme in the ergosterol pathway that is required for growth of the fungus. Not surprisingly, several mutations increasing azole tolerance were found in the gene body of resistant strains, however a further mechanism of resistance is a tandem repeat mutation in the promoter region[63]. This modification increases the transcription rate of the gene, resulting in higher protein concentrations to overcome the inhibitor activity of the fungicide. In azole containing compost heaps, 91% of ascospore-derived strains extracted were resistant, while in organic compost heaps only 2% were found to be resistant[64].

For a long time, the sexual cycle of *A. fumigatus* was unknown. Only recently was this process first observed, on oatmeal agar plates that are incubated in the dark at 30C for several months[65]. As previously highlighted, sexual spores (ascospores) of *A. fumigatus* present higher tolerance to temperature shocks, making it easy to isolate ascospores from parent tissues and the large amounts of conidiospores[65]. Previously, crosses in the presence of azole pressure between *A. fumigatus* tandem repeat strains have produced triple repeat strains which have higher resistance rates than double repeats[64]. However, little is known about whether the environment actively promotes recombination or passively selects resistant strains at the ascospore level.

Under azole pressure, wild-type strains show a higher expression of Cyp51 as well as another 185 genes after 4 hours in this environment[66]. Due to the nature of the tandem repeat mutation, azole-resistant strains may have a higher response capability to positive transcription factors that increase transcription further. Sexual crosses, rather than asexual propagation, appear to explain the formation and dispersal of these novel resistant varieties, as new resistant strains appear to accumulate previous mutations rather than originate from independent events[64].

If the hypothesis postulated previously holds, sexual crosses performed under azole stress environments will increase recombination specifically at this locus, preferentially at the promoter region of the gene. This recombination can produce novel tandem repeats at a higher rate, leading to azole resistance that can be inherited to the progeny. This can then be measured using DNA extracted from a pool of *A. fumigatus* recombinant progeny to detect the differences in recombination rate under different azole environments.

Materials and Methods

2.1 Pollen purification

This protocol is adapted from the method described by Choi *et al.* [59]. *A. Thaliana* inflorescences were collected in 50ml falcon tubes, immersed in 20ml 10% sucrose and stored overnight at -20C. This solution was thawed and blended using a waring blender with 3 pulses of 5 seconds each. The homogenate was filtered through 70nm nylon strainers. The concentration of pollen grains was determined using a Neubauer chamber. The suspension was centrifuged at 350g for 10min at 4C, after which the supernatant was decanted and the pellet resuspended in sucrose 10% solution. A further centrifugation step at 100g for 10min at 4C was used to purify the pollen grains further before a final count.

2.2 Characterization of crossover positions in *A. thaliana*

A list of genomic regions containing meiotic recombination positions was generated by combining the data obtained by Wijnker *et al.*[32] and Choi *et al.*[67]. Genes and non-coding RNA sequences contained within these regions, as well as information on the organ in which they are transcribed was obtained for each crossover position from the TAIR database (https://www.arabidopsis.org/). Transposon insertion sites were listed in the loci that did not contain annotated transcribed elements.

2.3 Purification of conidial DNA

Conidial spores of *A. fumigatus* were suspended in a 1.5ml eppendorf 500µl of breaking buffer solution consisting of 2% triton X-100, 1%SDS, 100mM NaCl, 10mM Tris HCl and 1mM EDTA at a pH 8 using 300mg glass beads of approx. 0.5mm diameter. After 10 seconds of thorough vortexing, suspensions were shaken at 1400rpm 30min at 70C. 500µl of phenol chloroform isoamylalcohol solution was added to the solution and shaken for a further 5min at room temperature. The resulting emulsion was centrifuged at 14000rpm for 5 min, from which the supernatant was extracted. Further purification was conducted using a Qiagen DNA purification kit.

2.4 Determination of mating type in *A. fumigatus* strains

A series of *A. fumigatus* strains including a diverse set of polymorphisms within the Cyp51A gene were cultivated on Malt Extract Agar (MEA) containing 1µg/ml Copper sulphate, and had genomic DNA extracted as described . Conidial DNA was amplified in a PCR containing 0.02U/µl GoTaq polymerase (Promega), 0,2mM dNTP mixture, 1x Green Gotaq buffer (Promega), 1mM $MgCl_2$ and 0.2pm of primers AFM1 AFM2 & AFM3 as described in Paoletti *et al.*[68]. The sizes of PCR products were verified by electrophoresis through a 1% agarose gel containing 50µM Ethidium bromide and a 1 kb DNA ladder (Promega). Products under 500bp were determined to be mating type 1-1 and products over 500bp were allocated to mating type 1-2.

2.5 Crossing and isolation of *A. fumigatus* ascospores

Strains V108-20, V108-21, 5-4, 39-3, 19-4, 99-1, H30 and H33 of *A. fumigatus* were selected for their promoter and gene body polymorphisms. Criteria of selection included mating type, type of tandem repeat and aminoacid polymorphism in the protein Cyp51A. All strains were crossed amongst each other on oatmeal agar containing 0, 0,25, 1 and 2 mg/l voriconazole at 30C for 3 months. Cleistothecia were extracted from the plate using an inoculation needle, cleaned on water agar 1% and dispersed in 300µl saline solution before suffering a heatshock treatment at 70C for 24h in a water bath. Serial dilutions of ascospores were made and plated on Malt extract agar (MEA) to quantify the ascospore concentration in solution and proceed to DNA extraction of conidia.

2.6 Sequencing of the Cyp51A gene region

Conidial DNA was amplified in a PCR containing 0.02U/µl Promega GoTaq polymerase, 0,2mM dNTP mixture, 1x Green Gotaq buffer, 1mM $MgCl_2$ and 0.2pM of each of a set of universal primers spanning the Cyp51A gene and the upstream intergenic region (ref for primers). Primers were either provided by Eveline Snelders, engineered from the primers used by Eveline for higher annealing temperatures with the Oligo analysis tool from Eurofins Genomics (https://www.eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/) or determined using the Primer3 webpage (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). PCR products were verified for size by electrophoresis through a 1% agarose gel containing 50µM Ethidium bromide. Products were either purified using a DNA purification kit (manufacturer) or mixed in a ratio of 2µl PCR product to 5µl ExoSAP-It, (manufacturer) and sent for sequencing to eurofins genomics. Sequences received were aligned using MUSCLE[69] to determine the presence of polymorphisms in the sequence.

2.7 Design of allele-specific primers

Primers with annealing temperatures of approximately 58C were designed to bind specifically on the 3' end to polymorphisms within the sequenced region using the Oligo analysis tool from Eurofins Genomics. Serial amplifications were conducted on a temperature gradient with 8 temperatures between 55C and 65C using the previous mix with a combination of an allele specific primer and a universal primer with a binding temperature of no less than 65C. The products of these amplifications were analysed using the agarose gels described previously to determine optimum annealing temperature.

Results

3.1 Purification of *A. thaliana* pollen

We aimed to develop a crossover-specific PCR as described by Choi *et al.*[59] to observe the difference in recombination frequency for selected genes in F1 *A. thaliana* strains. The major selection criterium was to be different transcription rates in the presence of different cytosolic genomes. The first stage in this protocol is to purify pollen grains for DNA extraction. The initial concern regarding the experiments was the sample size, as 37.000.000 pollen grains were necessary to prepare a sample for the crossover-specific PCR assay [59].

150 A. thaliana inflorescences from around 50 plants were blended in 10% sucrose solution and strained through a fine mesh. Pollen grains were counted with a Neubauer chamber, resulting in 2.1 million pollen grains per ml of solution. This solution was centrifuged at 350g for 10 min, the pellet resuspended and centrifuged at 100g for 10 min to concentrate the pollen. The total concentration of pollen was 18.5 million pollen grains in 5ml of resuspended solution, indicating a higher concentration in the pellet. This figure would indicate the required sample size for Arabidopsis plants in pollen typing experiments to be circa 100 plants, according to pollen counts. This would produce a final product of 100µl of DNA at a concentration of between 10 and 50ng/µl of template DNA depending on quality[59]. Depending on crossover numbers, a PCR assay can be performed with 1µl of this solution, thus providing ample material for experiments with 100-200 plants. Further centrifugation steps were not carried out due to the pollen used on this trial not being experimentally useful.

3.2 Annotation of recombinant loci in *A. thaliana*

In order to increase the chances of observing differential recombination rates at specific loci, the decision was made to analyse loci that had already been observed to present recombination in previous experiments. A list of 71 crossovers in pollen tetrads described by Wijnker *et al.* was used as a reference for this purpose. A further 2 loci were obtained from previous pollen recombination assays performed by Choi *et al.* [67]. The genes within the recombination loci were annotated, alongside information specifying the tissues where they were transcribed using the TAIR database.

Out of the 73 regions containing recombination sites, 57 of them contained a total of 86 coding sequences, including genes and long non-coding RNAs. 12 more were putative T-DNA insertion sites and 4 loci had no annotated elements in the reference genome. 42 of the 73 loci had genes transcribed in the “flower” category of tissues where transcription was located. As such, these genes could be transcribed in meiotic tissue within the flower. 8 more had genes with unknown transcription profiles and 7 contained genes that were not annotated as transcribed in ‘flower’. This included a locus with genes transcribed in plant sperm cells, another in the endosperm and embryo, 3 transcribed in stomatal guard cells and two in other organs not associated with reproduction. 78% of recombination events occur thus in regions containing coding sequences, and within those loci, 74% are transcribed in the reproductive organ of the flower. This follows well with the number of genes

3.3 Selection of *A. thaliana* loci for pollen typing experiments

Cytoswap lines of *A. thaliana* were chosen for analysis of recombination rate. As described previously, cytoswap lines contain nuclear genomes from one strain of *A. thaliana* and cytosolic genomes from another strain[61]. This produces a change in the transcriptome as compared to matching nuclear and cytosolic genome. By using this

form of stress, we can be certain that meiotic tissue will be suffering the same stress as the rest of the plant, as male meiocytes contain plastids[70].

The list of genes previously obtained was contrasted with transcriptome data from cytoswap lines developed by Flood and Theeuwen *et al.*[61]. Despite one of the genes being differentially transcribed in one of the cytoswap lines, and the crosses to observe that effect in the F1 made, the decision was made to not continue the experiment. Criteria such as number of genes differentially transcribed in the cytoswap lines having genome-wide consequences on recombination and the lack of consistency of the effect of cytosolic genomes on the homozygous cytoswap lines were considered in the decision.

3.4 Determination of mating type of *Aspergillus fumigatus* strains

A. Fumigatus Strain	Cyp51 Promoter repeat	Cyp51A mutations	Mating type
5-4	TR34	L98H	1-1
11-1	TR34	L98H	1-2
18-2	TR34	L98H	1-1
19-3	TR34	L98H	1-1
26-4	TR34	L98H	1-1
39-3	TR34	L98H/ S297 T-A	1-2
47-4	TR34	L98H/ S297 T-A	1-2
66-4	TR34	L98H	1-1
84-2	TR34	L98H	1-1
84-3	TR34	L98H	1-1
100-1	TR34	L98H	1-1
100-2	TR34	L98H	1-2
19-4	TR46	Y121 A-T/ T289 A-G	1-2
40-1	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-1
52-2	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-1
70-1	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-1
77-3	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-2
94-1	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-2
97-1	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-2
97-4	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-2
99-1	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-1
101-1	TR46	Y121 A-T/ T289 A-G/S363P T-C/, I364V A-G, G448S G-A	1-2
H30	$TR46^3$	Y121F/M172I/T298A/G448S	1-1
H33	TR46	Y121F/M172I/T298A/G448S	1-2
V108-20	WT	-	1-1
V108-21	WT	-	1-2

Table 1: List of Aspergillus fumigatus strains characterized by the type of repeat, mutation in Cyp51A gene and mating type. Mating type 1-1 indicates the presence of the MAT1-1 allele, which is sexually compatible with the MAT1-2 allele[64].

A. Fumigatus is an ascomycete fungus for which sexual reproduction has recently been discovered[65]. There is much interest in observing the influence of recombination on the evolution of an azole fungicide resistance, found in the environment. This resistance is based on the overexpression of the protein Cyp51A due to a tandem repeat mutation in the transcription promoter of the gene, which may have arisen from sexual recombination[64]. This same gene increases its transcription under azole stress[66]. If transcription rate correlates with recombination rate, there may be higher recombination rates under azole stress, increasing the speed of adaptation of the gene towards azole stress tolerance.

In order to determine which strains of *A. fumigatus* were to be crossed, a set of strains with known polymorphisms in the gene Cyp51A had to be tested for mating type. Mating type was determined by amplifying genomic DNA of these strains with the PCR and specific primers as described by Paoletti *et al.*[68]. Products of 438 bp determine mating type 1-1 and products of 834bp determine mating type 1-2. These were then run on an agarose gel to visualize the products, allowing us to determine the mating type (Table 1).

3.5 Crossing of *A. fumigatus*

A. fumigatus strains 5-4, 19-4, 39-3 and 99-1 were selected alongside the previously known strains V108-20, V108-21, H30 and H33 (table 2). Strains were selected for their promoter, mating type, type of tandem repeat and aminoacid polymorphisms in the protein Cyp51A. To derive control and test populations, the strains were crossed in all viable combinations on agar plates containing concentrations of 0, 0,25mg/l, 1mg/l & 2mg/l of voriconazole to incrementally stress the fungus, making a total of 64 plates. Plates were incubated for 4 months at 30C in the dark and checked fortnightly.

After 28 days, the 4 crosses between strains V108-20 and V108-21 and V108-20 and 39-3 at concentrations of 1mg/l and 2mg/l of voriconazole showed no growth, and thus were discarded. Strains V108-20 and V108-21 formed cleistothecia at concentrations of 0 and 0,25mg/l as well as strains H30 and H33. Further crosses at 0 mg/l of voriconazole had occurred between strains V108-20 and H33, and strains V108-21 and H30.

Cleistothecia from crosses involving strains H30 and H33 had a pale brown colour, distinct from the white colour of the crosses between wild-type strains. There were no further crosses for the duration of the experiment.At concentrations of 0,25mg/l and lower, *A. fumigatus* grew with a characteristic dark greenish gray, covering the whole plate except in the cross between wild type strains at 0,25mg/l. At higher concentrations of voriconazole, the colonies were a pale brown colour with the crosses between strain 5-4 and both V108-21 and 39-3 presenting amorphic structures, larger at the higher 2mg/l voriconazole concentration.

To extract DNA for evaluation of crossover frequency in *A. fumigatus* sexual progeny, Cleistothecia were harvested from all successful crosses using an inoculation needle. To ensure all parental conidiospores did not contribute to the population, samples were heatshocked at 70C for 24 hours, killing all parental tissue[64]. Ascospores were plated on Malt extract agar (MEA) under different solution concentrations to ensure well defined single-spore colonies. All the progeny from sexual crosses gave growth of A. fumigatus colonies with dark green conidia, indicating healthy progeny[65].

0 Voriconazole			1-2			
			V108-21	39-3	19-4	H33
			WT	TR34	TR46	TR46^3
1-1	V108-20	WT	**1**	**2**	**3**	**4**
	5-4	TR34	**5**	**6**	**7**	**8**
	99-1	TR46	**9**	**10**	**11**	**12**
	H30	TR46^3	**13**	**14**	**15**	**16**

0,25mg/l Voriconazole			1-2			
			V108-21	39-3	19-4	H33
			WT	TR34	TR46	TR46^3
1-1	V108-20	WT	**1**	**2**	**3**	**4**
	5-4	TR34	**5**	**6**	**7**	**8**
	99-1	TR46	**9**	**10**	**11**	**12**
	H30	TR46^3	**13**	**14**	**15**	**16**

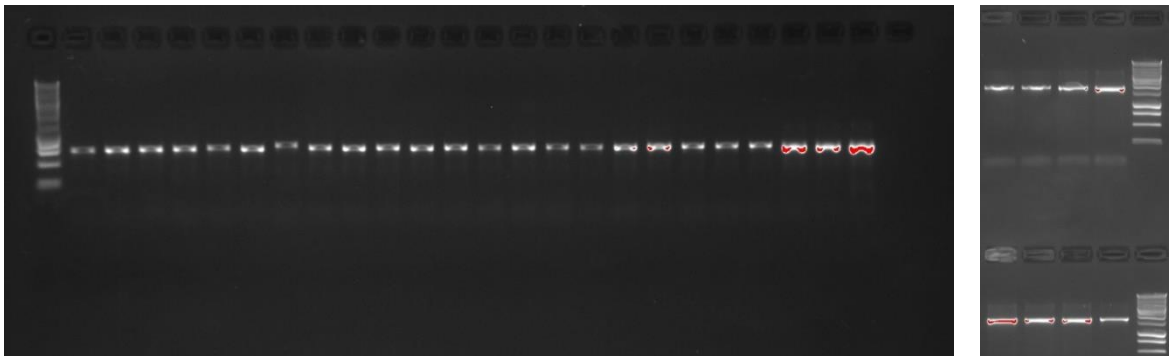
1mg/l Voriconazole			1-2			
			V108-21	39-3	19-4	H33
			WT	TR34	TR46	TR46^3
1-1	V108-20	WT	**1**	**2**	**3**	**4**
	5-4	TR34	**5**	**6**	**7**	**8**
	99-1	TR46	**9**	**10**	**11**	**12**
	H30	TR46^3	**13**	**14**	**15**	**16**

2mg/l Voriconazole			1-2			
			V108-21	39-3	19-4	H33
			WT	TR34	TR46	TR46^3
1-1	V108-20	WT	**1**	**2**	**3**	**4**
	5-4	TR34	**5**	**6**	**7**	**8**
	99-1	TR46	**9**	**10**	**11**	**12**
	H30	TR46^3	**13**	**14**	**15**	**16**

Table 2: Crosses made between *A. fumigatus* strains. Mating type, indicated as 1-1 and 1-2, and promoter repeat type is listed for each strain. Successful crosses are highlighted in green, red squares mark crosses that did not grow on plates.

3.6 Sequencing of *A. fumigatus* strains

In order to develop a crossover specific PCR, it is necessary to find polymorphisms between the parental sequences to design allele-specific primers. For this purpose, the Cyp51A gene body and upstream DNA for each strain was sequenced to determine polymorphisms between the strains. Genomic DNA from *A. fumigatus* parental conidia was purified using a previously described technique[64]. Promoter regions and gene regions were amplified separately and sequenced bidirectionally. Reads were aligned to produce continuous sequences for each strain using MUSCLE[69]. Gene sequences of strains V108-20, V108-21, H30 and H33 were aligned with one another to detect SNPs between strains. The promoter sequences confirmed the presence of tandem repeats, with the only surprise coming from strain H33, which was shown to be only a double repeat after multiple sequencings. However, between the strains, there were no further polymorphisms in the promoter regions. Further sequencing upstream of the wildtype V108-20 and V108-21 strains and triple repeat strains up to 2kb from the transcription start site of the Cyp51A gene revealed further identical sequence. SNPs thus were only detected in the gene-coding region of the Cyp51A gene (Fig. 6).



A B

Fig.5: A. Promoter amplicons using primers pA7 and 365.12 from *A. fumigatus* strains. B. Coding region amplicons using primers from left to right above with DNA from strains 108-20, 108-21, 5-4, 19-4, and from left to right below 39-3, 99-1, H30 and H33.

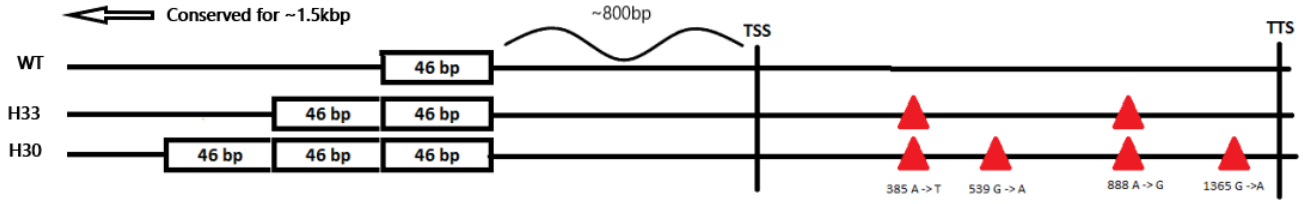


Fig.6: Scheme indicating the polymorphisms between WT *A.fumigatus* as represented by strain V108-21, and strains H33 and H30. SNPs are marked with red triangles, and mutation is indicated below. Tandem repeats are indicated as rectangles.

3.7 Allele-specific primer design

In order to develop a crossover-specific PCR to measure recombination at the Cyp51A locus, allele-specific primers were designed for the SNPs identified in the gene body regions between strains H30 and H33 and the wild-type strains and H30 (Fig.6). Criteria for design were placing SNP positions on the 3' end and annealing temperatures of approximately 58 °C, alongside checking for dimerization. This resulted in 8 primers for SNPs between wild-type strains and H30, and 4 primers between strains H30 and H33 (table 4).

Table 4: List of designed allele-specific primers. Primers were named according to the approximate location of the polymorphism according to the TSS, intended direction of amplification and the specific strain for which the primer was designed. Bases binding to polymorphisms between the parental strains are marked in red.

Primers for cross 108-21 x H30	
410F 108-21	5′-ATCGGACGTGGTGTA-3′
410F H30	5′-ATCGGACGTGGTGTT-3′
410R 108-21	5′-CAGCTTGGAATTGGGACAATCAT-3′
410R H30	5′-CAGCTTGGAATTGGGACAATCAA-3′
560R 108-21	5′-CATTGCCGCAGCGATGTCC-3′
560R H30	5′-CATTGCCGCAGCGATGTCT-3′
910R 108-21	5′-GACCAGCCATCAACAGGGT-3′
910R H30	5′-GACCAGCCATCAACAGGGC-3′

Primers for cross H30 x H33	
565R H30	5′-CATTGCCGCAGAGATGTCT-3′
565R H33	5′-CATTGCCGCAGAGATGTCC-3′
1390R H30	5′-GGTGTCGGCCAGCACT-3′
1390R H33	5′-GGTGTCGGCCAGCACC-3′

Allele-specific primers for strains H30 and H33 were tested for specific amplification using primers with high annealing temperatures (>65C) previously used for sequencing. A temperature gradient from 55C to 65C was used to find the optimal annealing temperature for the different primers and determine their specificity. The primers 1390RH30 and 1390RH33 proved to be too unspecific for use due to the presence of more than a single band in each amplification, while the primers 565RH30 and 565RH33 appear to dimerize in combination with different forward primers, with intense bands below the 250bp band of the 1kb DNA ladder (Fig.7).

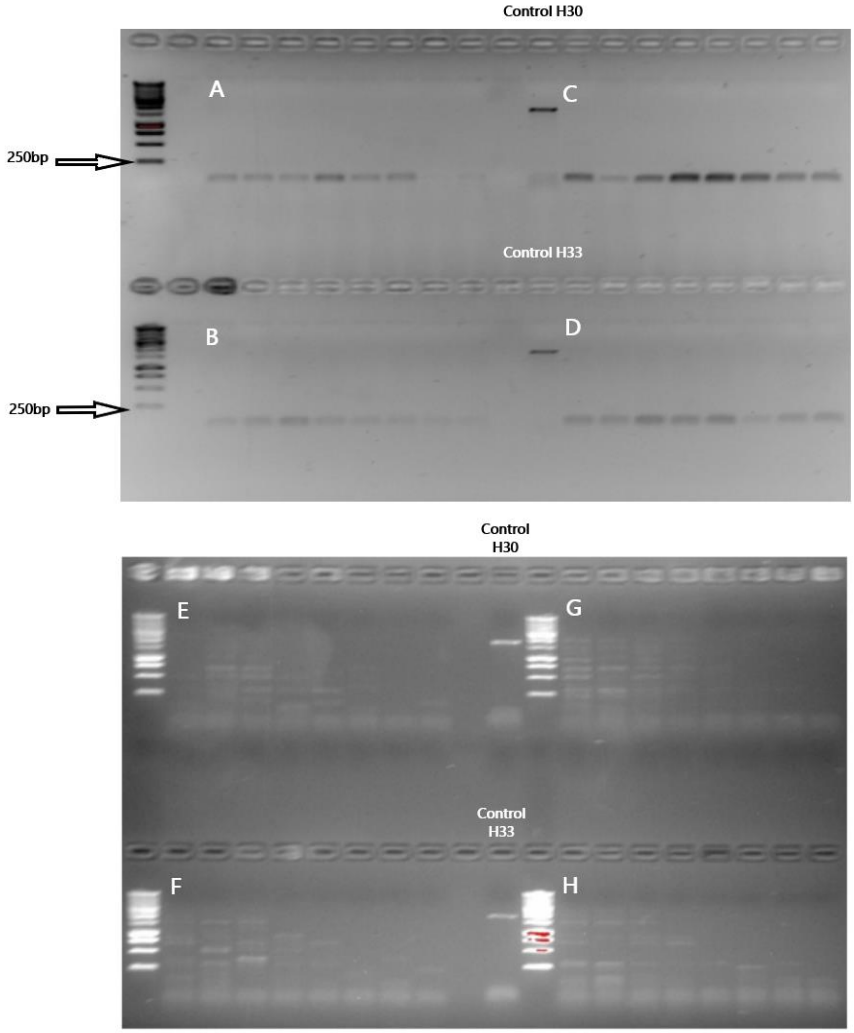


Fig.7: UV photographs of 1% agarose gels containing the PCR products from genomic DNA of strains H30 (A,C,E,G) and H33 (B, D, F, H) amplified with primers 565RH30 (A, B), 565RH33 (C, D), 1390RH30 (E, F) and 1390RH33 (G,H). All samples shown were amplified using the forward primer 365.1+. For each combination of genomic DNA and primer, reactions were carried out at a gradient of 8 different temperatures, ranging from 55°C on the left to 65°C on the right. Note that the primers binding to the polymorphism at position 539 give products smaller than 250bp where a product of ~550bp is expected, and primers designed for the polymorphism at 1365 show multiple

Discussion

There is an abundance of circumstantial evidence that links transcription and meiotic recombination hotspots, impacting the genetic makeup of the next generation of organisms. For many years however, the studies addressing this link have focused on limited regions of the genome, without taking the entire genome transcription dynamic into account. Furthermore, the difficulty of analysing recombination rates at the gene scale is manifest due to the low probability of these events in short regions. In this project, my aim was to implement a protocol previously described by Choi et al.[59] to analyse crossover rates in short kilobase pair sized regions and use it to study recombination rates in different transcription environments. I implemented a pollen-isolation protocol in Arabidopsis, that can be used to set up recombination experiments in Arabidopsis. After due consideration of the challenges of inducing a targeted transcription increase in *Arabidopsis thaliana* meiosis, I attempted to develop the technique in the ascomycete *Aspergillus fumigatus.*

For setting up the experiments as proposed, there are several requirements to be met. These include the number of meiotic spores required for analysis, the manipulation of environmental conditions during spore generation and the presence of sufficient polymorphisms in the region of interest. I will discuss these and others in the following section, after which I will elaborate on alternatives to address the hypothesis associating recombination to transcription.

4.1 Sample size determination

The first step for developing a crossover-specific PCR protocol is to evaluate how much progeny necessary to observe a significant effect. Determining this number is necessary to generate sufficient F1 progeny for experiments containing control and treatment sample populations. In *A. thaliana*, we determined that a replicate sample size of 100-150 plants is sufficient to produce enough pollen for repeated measurements. This means that an experiment containing 3 replicates of a control population and 3 replicates of a stress treatment would require circa 900 plants, making it a feasible experiment for the scope of a Master thesis. However, the only way to ensure a significant result is to observe the number of recombination events at the locus.

In *A. fumigatus*, each cleistothecium produces ascospores in the order of 10^4 to 10^5 asci, and hundreds of cleistothecia growing on a single plate of oatmeal agar in the more fertile crosses[71]. Furthermore, the recombination frequency of the related *Aspergillus nidulans* is between 55 and 80 crossovers per meiotic division within a 30Mb genome[72], as compared to 11 crossovers in pollen between 130 Mb of *A. thaliana* genome[32]. This increased recombination rate per individual increases the likelihood of significant differences between recombination rates under different environments with the same amount of progeny. As such, the experiment using *A. fumigatus* as a test subject is also practical in the scope of an MSc. Thesis.

4.2 Meiotic crossing conditions

Also important for the technique to succeed is the easy generation of F1 progeny from two divergent strains. The technique for generating *A. thaliana* hybrids is well established: it takes around two months to produce viable progeny between different varieties. However, applying stresses to meiotic tissues in plants is not as straightforward as in other tissues, as these are isolated from the environment in organs lined with somatic tissue. It is known that temperature and developmental age can have an effect on meiotic recombination hotspots, indicating an impact of the environment on the meiotic cell[38]. However, transcriptome analysis techniques have only recently been developed for plant meiotic tissues[73], and trials have not yet been

conducted in stressful conditions. As such, the impact of other stresses on recombination is not so clear. Defence hormone signalling does impact the development of flowering[74], however the manner in which this affects the transcription profile of the flower requires further study.

In *Aspergillus spp.,* the exposure of reproductive tissue to the environment is more direct[75]. Meiotic cells are not insulated by somatic tissue and thus may respond to environmental stresses themselves. However, meiosis in *A. fumigatus* is severely restricted to a specific environment[65]. Our experiments show that it is possible to cross *A. fumigatus* at low concentrations of voriconazole as a part of this specific environment. However, we did not have any crosses at highly stressful concentrations, highlighting the restrictions for meiotic division aforementioned.

Furthermore, there were no crosses between wild-type lines and resistant lines in voriconazole-containing medium. This could be due to resistant strains outgrowing and displacing susceptible ones, reducing the number of opportunities for crossing. This hypothesis is supported supported by the presence of amorphic structures at higher azole concentrations in crosses 5 and 6 which disappeared in crosses 7 and 8, despite the same strain being present. The degree by which resistant strains outcompete wild-type strains would have to be tested by quantitatively genotyping the strains growing on the plate. As such, the chances of obtaining a satisfactory result would be larger using an organism with an established record of sexual crossing with environmentally exposed tissue in numerous environments.

4.3 Polymorphism abundance

The next stage in the development of a crossover-specific PCR is detecting polymorphisms that can be used as allele-specific primer binding sites. By using sequenced *A. thaliana* lines with well-known specific markers[32], we could quickly identify regions of interest and design universal primers for quick testing of candidate primers. This information was not available for the *A. fumigatus* strains that we used, however given the number of non-synonymous mutations present in the strains studied[64], we anticipated there would be further synonymous polymorphisms.

This assumption proved to be erroneous, as the results from the sequencing show that there is a high degree of conservedness for the CYP51 gene between the strains that were able to cross in the four-month period that these crosses were attempted. Cyp-51A is an essential gene necessary for the development of the cell wall and is thus highly transcribed in wild-type *A. fumigatus* strains[66]. It is likely that the increased transcription rate in the promoter tandem repeat increases codon selection pressure in the gene. The phenomenon of codon-adaptation is well established in highly transcribed genes, with gene sequences in these genes under selection pressure for tRNA ratios in the cytosol[56].

Surprisingly, the sequencing data highlighted the absence of SNPs in the region 2kb upstream of the CYP51 gene between the different strains. This contrasts with the gene-coding region, which presented a small number of SNPs, indicating strong conservative selection for the promoter region. This conserved region was thereafter confirmed for at least 10kb between the wild-type lines by whole genome sequencing data obtained from Joost van den Heuvel (unpublished). It is reasonable to assume a high degree of selection, due to the fast growth rate and abundant conidia being selected against mutations in the region. An increased recombination rate in this region would reduce the degree of linkage with contiguous loci, allowing for a stronger selection while increasing the amount of viable progeny and reducing the selection pressure on contiguous loci.

4.4 Quality of polymorphisms for strain-specific primer design

The next stage of the development of the crossover-specific primer was the design of Allele-specific primers for the SNPs found. This was done for most of the SNPs found between the strains that crossed. However, in the region explored, there were not enough polymorphisms to develop a full nested PCR as prescribed in the protocol. Primer specificity depends highly on the nature of available polymorphisms. Ideally, allele-specific primers bind to short deletions to maximise the specificity[59]. Alternatively, they can contain multiple SNPs in a short region. Due to the conserved nature of the sequences used In *A. fumigatus*, only single SNPs were available for primer design. This limited the specificity of the allele-specific primers available to individual mismatches at the 3'end of the primer. These primers proved to be unsatisfactory for use in an allele-specific PCR trial due to either lack of specificity or lack of adequate primer binding.

These two issues highlight the fundamental need for an abundant set of polymorphisms, including either indels or clusters of SNPs, to develop an allele-specific PCR. A minimum of 4 polymorphic regions are necessary to develop a nested PCR, but taking into consideration the potential for unspecific primers it is preferable to have a larger number of SNPs to ensure success. This region can be extended as long as a reliable PCR product can be generated, which in the case of commercial PCR mixes can extend as far as 20kbp. However, in the case of Cyp51, data obtained from whole-genome sequencing indicated a large region of at least 10kb devoid of SNPs between the 2 wild-type strains, and resistant strains may have presented similar levels of conservation.

Studies observing the evolution rate of essential and non-essential genes in *S. cerevisiae* discovered to their surprise that essentiality is not an influential factor. In fact, the only significant factor related to evolutionary rate was gene expression, with less expressed genes showing higher mutation rates[57]. Cyp51A is a gene essential for *A. fumigatus* growth, and thus is highly expressed[66], likely contributing to the higher degree of sequence conservation.

Defence gene transcription is often triggered by the environment and is thus often silent in environments where the genes are not required. Defence genes were found in Drummond *et al.* to have the fastest evolution rate between *S. cerevisiae* strains[57]. Thus, studying genes related to defence response may be beneficial not only for large divergence in transcription under different environments, but also for increased chance of finding sufficient polymorphisms. The region chosen by Choi *et al.* to apply the crossover-specific reflects this phenomenon by containing many genes related to defence in *A. thaliana* [67].

4.5 Choice of transcription modulation technique

There are multiple approaches available to alter the expression rate of a gene. In this thesis, I attempted to use environmental stressors such as cytosolic perturbation and exposure to fungicide treatment to alter the transcriptome of cells undergoing meiosis. Using external stressors has the advantage of comparing identical genomes and underpinning an environmental influence to meiotic crossover positioning. It is often technically simple to apply an extrinsic stress such as temperature or toxin exposure to an organism. However, using this strategy also has its downsides. First and foremost, environmental stresses may restrict or even impede sexual reproduction. Stresses must therefore be carefully dosed to induce gene transcription without excessively reducing the production of sexual spores.

It is also important to note that environmental stresses may alter the transcription of many genes. The phenomenon of crossover homeostasis restricts an increase in number of crossovers within a specific chromosome[26]. It is therefore likely that a global increase in gene transcription rate would not lead to a

proportional increase in crossover events. In the case the hypothesis relating transcription and recombination holds true, the effects of transcription increase would be dispersed between the number of genes presenting increased transcription.

In this project, we observed the global effect of cytoswap strains on the *Arabidopsis* genome. We observed that changing the plasmotype changed the expression profile of a large number of genes, diluting the increase in recombination amongst many loci. This would increase the sample size necessary to observe significant effects. This global increase in transcription may be the phenomenon producing experimental results that contradict the hypothesis here proposed due to application of genome-wide alterations and measurement of recombination at specific loci. On the other hand, stresses that increase transcription for a limited pool of genes will produce larger effects on those loci that can be more easily measured. Developing experimental approaches that lead to the specific upregulation of transcription in a limited number of loci would therefore be most useful.

Another option available is to knock out specific transcription factors modulating expression. Transcription factor binding sites for specific inducers and suppressors of transcription are known to be associated with recombination hotspots[32]. Knocking down a transcription factor is an effective way to suppress transcription in a specific locus[76]. However, this does have the disadvantage of comparing organisms with different genomes, and transcription factors can have a large effect on the transcription profile of many genes, leading to the same issues discussed previously. Also, the transcription factor knockout must be bred into both parental lines, making the setup of the experiment a time-consuming process. A further argument can be made that transcription factors may be themselves involved in recruiting recombination machinery regardless of their effect on transcription.

Crispr-Cas gene editing can be used to modify transcription rates by editing transcription factor binding sites. This induces transcription modulation at the locus of interest without modifying transcription rates in the rest of the genome. Furthermore, gene edits can be designed such as to provide allele-specific primer binding sites, facilitating the development of a crossover-specific PCR assay. Arguably, modification of the sequence at the promoter region may influence crossover positioning due to currently unknown causes. Ideally, genes with few known interactions should be chosen to limit the global effects on the organism's homeostasis. The process of creating Crispr-Cas modified lines is lengthy and would thus require careful planning to increase the chances of success.

Finally, transcription may also be induced by knocking out or reducing the efficiency of stress-related proteins and applying moderate levels of stress. In *A. thaliana* and *S. cerevisiae,* transcription factors involved in defence and oxidative stress response often work via a feedback loop. If the response genes perform their function, the signal inducing transcription is repressed, leading to the repression of transcription of the response genes. If the defence response is not completely efficient however, transcription can become chronic[7747]. This signal, alongside the synthesis of hormonal signals, may have an influence on the transcriptome of meiotic cells[74].

4.6 Alternative options for exploring the association between crossover events and transcription

With current technology, it is impossible to observe crossover events at a lower resolution than at the polymorphism level. As such, crossover-specific PCRs are the most effective way of quantifying recombination events in short regions presenting a small number of crossover events. However, developing a working protocol with allele-specific primers for assessing the recombination frequency at a specific locus involves a lot of work as we have seen in this project. The choice of the gene is critical for determining the chances of success, as a

locus without a sufficient number of viable polymorphisms cannot be used in this assay. It is also important for it to respond to transcription modulation without a large number of other loci following the same transcription pattern.

In larger genomic regions however, the chance of finding a crossover event is proportionally larger. There is much research describing the phenomenon of genes with similar expression patterns clustering at specific genome positions. This is especially the case in ascomycetic fungi, which often present gene clusters involved in secondary metabolite synthesis. These clusters remain inactive unless triggered by environmental stresses, thus facilitating the design of experiments under different environments. Crossover events in these regions would have a high impact on the segregation of the gene cluster components, increasing the speed of adaptation to the environment.

To detect these phenomena, Whole Genome Sequencing (WGS) could be used as specified by Wijnker et al. This forfeits the need for primer design by extracting the whole sequence from each organism. Crossover events would be detected between individual SNPs. The resolution of this detection is determined by increasing the sequence density. However, this degree of resolution is not necessary for detecting crossover events in large regions. With the relatively low price of sequencing, low resolution sequencing can be performed on large numbers of progeny for a relatively low price. This is especially true for organisms with a small genome size such as *S. cerevisiae* and *A. nidulans*.

By coupling this technique with high-throughput RNA sequencing, a complete picture of the transcription landscape can be superposed onto the genome sequence. The hypothesis would be confirmed if recombination rate divergence determined by WGS were associated with regions of higher transcription under different environments. Furthermore, superposing the sequences of the progeny would produce in itself a high-resolution genome sequence of the two parental strains, facilitating the identification of polymorphism-rich regions for in-depth study using crossover-specific PCRs. If there happens to be no correlation between transcription and recombination, this relationship will be demonstrated to be false, and focus should be redirected to other explanations for the variability in meiotic crossover landscapes.

Conclusion

Due to the lack of SNPs in the Cyp51A gene of *A. fumigatus* and the large number of genes differentially expressed in cytoswap arrays, it has been impossible to develop a crossover-specific PCR within this project. However, the feasibility of the procedure is manifest, especially in *A. fumigatus,* with adequate selection of gene targets to analyse. Sadly, no results addressing the hypothesis of gene transcription enhancing recombination have been produced but will hopefully be performed in the not too distant future.

In this thesis, I propose three major arguments in favour of the association between transcription and recombination. Firstly, the transcription-related phenomena surrounding crossover hotspots and their localization proximal to transcription start sites. Secondly, the modulation of hotspot positioning in different environments coupled with genome clustering may favour the suppression of recombination between positively interacting genes and enhance recombination at negatively interacting clusters, speeding up adaptation. Finally, it is an explanation for the low mutation rate of highly expressed, non-essential genes, which is currently being explained with hypothesis regarding selection pressure of efficiencies and codon proportions.

Recombination is a balance between keeping together genes that work and incorporating new combinations that ensure survival against random deleterious mutations and permit growth in novel environments. The most beneficial distribution of recombination is thus far from random and depends on the stresses that the organism is facing. Transcription is a good indicator for an organism that it is time to refresh the gene compositions that were adequate in the past, minimising the amount of inviable progeny and taking evolutionary risks where they are most beneficial.

Currently however, breeding programs assume random recombination between genes, and nurse thousands of plants in the hope that they find the recombination event that they want. By having a deeper understanding of the mechanisms behind recombination, these programs can become more efficient, increasing the speed at which disease-resistant strains can be developed. If transcription is indeed related to recombination, this may explain the relative difficulty in breeding stress resistances, as current breeding practice involves growing in ideal environments to maximise the amount of progeny. Potentially, applying moderate amounts of stress may induce transcription enough to increase the chance of recombination close to stress-response genes, increasing segregation and speeding up the breeding process.

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Late in 2018, I had just finished GATC in order to work with cytoswap strains under Tom Theeuwen, a project which eventually fell through. I was and am interested in plant breeding for stress resistances, and thought cytoswap study was an interesting way of exploring the way in which plant genes interact. I thus was attracted to the Chromosome substitution lines (CSL) developed by Erik Wijnker.

The embryo of this project was proposed to me after several discussions regarding CSL lines. Erik was interested in the divergent recombination patterns between male and female *A. thaliana* gametes, and hypothesized that epistatic interactions could be involved in favouring divergent recombination landscapes. The bumps, fumbles and mistakes were more than worth the opportunity to explore the realm of recombination without a safety harness tugging me back, and if anything, I enjoyed the conversations too much. I also appreciate the extensive comments I received for many different texts I produced, highlighting the

After we got stuck with the *A. thaliana* experiments, Fons Debets kindly heard out our hypothesis and pointed us to Eveline Snelders, who was studying the influence of recombination on the evolution of *A. fumigatus* resistances. The enthusiasm with which this new avenue of research was taken up was quite flattering, and it allowed us to have the first insights on the development of a crossover- specific PCR assay. I appreciate the time which Eveline dedicated to teaching me the ropes of handling *A. fumigatus*, and the patience expressed as I identified water droplets as cleistothecia in the early days.

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