QTL Analysis of High Thermotolerance with Superior and Downgraded Parental Yeast Strains Reveals New Minor QTLs and Converges on Novel Causative Alleles Involved in RNA Processing

Yudi Yang1,2, Maria R. Foulquié-Moreno1,2, Lieven Clement3, Éva Erdei1,2, An Tanghe1,2, Kristien Schaerlaeckens1,2, Françoise Dumortier1,2, Johan M. Thevelein1,2*

1 Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, KU Leuven, Flanders, Belgium, 2 Department of Molecular Microbiology, VIB, Leuven-Heverlee, Flanders, Belgium, 3 Department of Applied Mathematics, Computer Science and Statistics, Ghent University, Flanders, Belgium

Abstract

Revealing QTLs with a minor effect in complex traits remains difficult. Initial strategies had limited success because of interference by major QTLs and epistasis. New strategies focused on eliminating major QTLs in subsequent mapping experiments. Since genetic analysis of superior segregants from natural diploid strains usually also reveals QTLs linked to the inferior parent, we have extended this strategy for minor QTL identification by eliminating QTLs in both parent strains and repeating the QTL mapping with pooled-segregant whole-genome sequence analysis. We first mapped multiple QTLs responsible for high thermotolerance in a natural yeast strain, MUCL28177, compared to the laboratory strain, BY4742. Using single and bulk reciprocal hemizygosity analysis we identified MKT1 and PRP42 as causative genes in QTLs linked to the superior and inferior parent, respectively. We subsequently downgraded both parents by replacing their superior allele with the inferior allele of the other parent. QTL mapping using pooled-segregant whole-genome sequence analysis with the segregants from the cross of the downgraded parents, revealed several new QTLs. We validated the two most strongly linked new QTLs by identifying NCS2 and SMD2 as causative genes linked to the superior downgraded parent and we found an allele-specific epistatic interaction between PRP42 and SMD2. Interestingly, the related function of PRP42 and SMD2 suggests an important role for RNA processing in high thermotolerance and underscores the relevance of analyzing minor QTLs. Our results show that identification of minor QTLs involved in complex traits can be successfully accomplished by crossing parent strains that have both been downgraded for a single QTL. This novel approach has the advantage of maintaining all relevant genetic diversity as well as enough phenotypic difference between the parent strains for the trait-of-interest and thus maximizes the chances of successfully identifying additional minor QTLs that are relevant for the phenotypic difference between the original parents.

Introduction

Many genetic traits are quantitative and show complex inheritance. Because these traits are so prevalent in nature, understanding the underlying factors is important for various biological fields and for applications like industrial biotechnology and agricultural practice [1]. Recently, baker’s yeast *Saccharomyces cerevisiae* has become an important subject for studies in quantitative genetics [2,3]. In particular the availability of high-density genetic markers, the ease of performing experimental crosses and the powerful technologies for precise genetic modification [4,5], do not only allow efficient QTL mapping but also rapid identification of causative genes and their experimental validation and interaction analysis. *S. cerevisiae* displays many quantitative traits that are also important in other cell types, including industrial microorganisms and cells of higher, multicellular organisms. Such properties include thermotolerance [6] and oxidative stress tolerance [7], the capacity to produce small molecules, such as acetic acid [8] and ethanol tolerance [9,10]. Other quantitative traits that have been studied in yeast include transcriptional regulation [11], sporulation efficiency [12], telomere length [13], cell morphology traits [14], mitochondrial genome instability [15], global gene expression [16], evolution of biochemical pathways [17] and resistance to chemicals [18].

A major remaining challenge in quantitative trait studies is the efficient mapping of minor quantitative trait loci (QTLs) and
Identification of their causative genes. Minor QTLs have a subtle influence on the phenotype, which is easily masked by epistasis [19], gene-environment interactions [20], low association to the phenotype because of limited sample size and complex interactions with other QTLs. Minor QTLs are important because together they can produce in an additive or synergistic manner equally dramatic effects on the phenotype as major QTLs. Actually, the work of Bloom et al. [21], in which a large panel of individually genotyped and phenotyped yeast segregants was used, has shown that for 46 quantitative traits, the assembly of all detected loci can explain nearly the entire additive contribution to the heritable variation. The minor QTLs identified should be truly relevant for the trait of interest in the original parent strains and not generated in some unrelated way during the mapping analysis now allows successful identification of underlying genetic loci with a major effect. On the other hand, identification of loci with a minor contribution remains a challenge. We now present a methodology for identifying minor loci, which is based on the finding that the inferior parent usually also harbours superior alleles. This allowed construction for the trait of high thermotolerance of two ‘downgraded parent strains’ by replacing in each parent a superior allele by the inferior allele from the other parent. Subsequent mapping with the downgraded parents revealed new minor loci, which we validated by identifying the causative genes. Hence, our results illustrate the power of this methodology for successfully identifying minor loci determining complex traits and with a high chance of being co-responsible for the phenotypic difference between the original parents.

Results

Identification of QTLs determining high thermotolerance

We have screened a total of 305 natural and industrial isolates of S. cerevisiae for their ability to grow at high temperature, i.e. 40–41°C, on solid YPD plates. Not a single yeast strain was able to grow with a reasonable rate at 42°C. The strain MUCL28177 showed very good growth at 41°C and was chosen for further analysis. After sporulation, we selected a haploid segregant MUCL28177-21A, further referred to as 21A, which also showed excellent growth at 41°C compared to the control strain BY4742. Strain 21A was crossed with the laboratory strain BY4742, that is, excellent growth at 41°C. The hybrid 21A/BY4742 diploid strain grew at least as well as the 21A strain at 41°C, indicating that the high thermotolerance of 21A is a dominant characteristic. Phenotyping of 950 segregants of the 21A/BY4742 diploid strain revealed a range of thermotolerance. It resulted in 58 segregants with similar growth at high temperature as 21A. The growth of the original strain MUCL28177, the parent strains 21A and BY4742, the hybrid diploid strain 21A/BY4742 and ten representative segregants with varying thermotolerance, is shown in Figure 1.

The 58 thermotolerant segregants were pooled based on dry weight and genomic DNA isolated from the pool. Genomic DNA samples from the pooled segregants and from parent strain 21A were sequenced. The sequence reads obtained were aligned with the sequence of the reference S288c genome, which is essentially the same as that of the inferior parent strain BY4742. A set of quality-filtered SNPs to be used as genetic markers, was acquired essentially as described before [10]. For each chromosome, the SNP variant frequency was modeled using an additive logistic regression model [10,25]. The results are shown in Figure 2. In the top panel, the raw SNP frequencies are plotted against the
chromosomal position along with the modeled frequency (smoothed lines). The middle panel shows contrasts between selected pools and an unselected pool along with 95% simultaneous confidence bands. Upward and downward deviations from 0 indicate putative QTLs containing causative alleles from the superior and inferior parent, respectively. Normally, only linkage with the superior parent strain is expected. However, since the original MUCL28177 diploid strain is a natural isolate, it is likely heterozygous. Hence, the 21A segregant may contain recessive mutations that compromise to some extent thermotolerance in spite of the fact that its overall thermotolerance was only slightly lower than that of the MUCL28177 parent strain.

We calculated 2-sided p-value profiles along the chromosome that were adjusted for multiple testing (Text S1 online: Supplementary Methods) and five regions show significant p-values (0.05 significance level, Figure 2). We chose four regions with the smallest p-values for further analysis (Table S1 online). For these loci, selected SNPs were scored in individual thermotolerant segregants (up to 62 after additional segregant isolation and phenotyping) and a binomial exact test with FDR adjusted p-values was used for assessing statistical significance [10,26]. Three QTLs (QTL1, QTL2 and QTL3) were confirmed to exhibit statistically significant linkage to the high thermotolerance phenotype (0.05 FDR level, Table 1). QTL1 and QTL2 showed linkage with the genome of the superior 21A parent strain, while QTL3 showed linkage with the genome of the inferior BY4742 parent strain. We concentrated our work first on QTL1 and QTL3, because they showed the strongest linkage to

Figure 1. Thermotolerance of the parent strains and segregants. The diploid strain MUCL28177 was identified as a highly thermotolerant strain, showing strong growth at 41°C. One of its haploid segregants MUCL28177-21A (referred to as 21A) also showed high thermotolerance, whereas the control laboratory strain BY4742 did not grow at all at 41°C. The hybrid diploid strain 21A/BY4742 grew nearly as well at 41°C as its superior parent 21A, indicating that the major causative allele(s) in 21A is (are) dominant. The haploid segregants from 21A/BY4742 show varying growth ability (as indicated by a score from 0 to 6 for growth in the different dilutions) at 41°C, between that of the BY4742 inferior and 21A superior parents, indicating that thermotolerance is a quantitative trait.

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Figure 2. Genetic mapping of QTLs involved in thermotolerance by pooled-segregant whole-genome sequence analysis. Genomic DNA samples were extracted from an unselected pool (pool 0) and two pools of thermotolerant segregants able to grow at 41°C (Pool 1) and at 40.7°C (Pool 2), respectively. The DNA for each pool originates from 58 segregants. Pool 1 consists of segregants from the cross between parents 21A and BY4742 and Pool 0 and Pool 2 from the cross between the downgraded parents 21ADG and BY4742DG. The top-panel represents the SNP variant frequency.
the superior and inferior parent, respectively. The subtelomeric regions often show deviations from the 50% value of the SNP variant frequency, but this is also observed in the unscored pool. It may be caused by complications with the mapping of repetitive sequences, which are known to be commonly present in subtelomeric regions. We have analysed for instance the right subtelomeric region of chromosome X, in the mapping with the original parents, using SNP detection in the individual segregants and found a p-value that failed to indicate significant linkage (results not shown).

Identification of the causative gene in QTL1

We first fine-mapped QTL1 by scoring eight selected SNPs in individual thermotolerant segregants, which reduced the size of the locus to about 60,000 bp (Figure 3A). Detailed analysis of the 21A sequence of this region showed that 22 out of the 33 genes and putative ORFs present contained at least one non-synonymous mutation in the ORF compared to the BY4742 sequence (Figure 3A). Next we applied reciprocal hemizygosity analysis (RHA) [6] to identify causative gene(s) in QTL1. RHA is used to test for a possible contribution to the phenotype of each allele of the candidate gene in a hybrid genetic background. For each of the 22 genes with non-synonymous mutations, we constructed two 21A/BY4742 hybrid strains in which either the 21A or the BY4742 allele was deleted, so that each strain only contained one specific allele of the candidate gene. Comparison of the growth at high temperature (41°C) of the two hybrid strains did not show any difference for the 22 candidate genes, except for MKT1 (Figure 3B, Figure S1 online and data not shown). The hybrid strain with the MKT121A allele showed better growth than the strain with the MKT1BY4742 allele. We further confirmed the relevance of MKT1 by demonstrating that MKT1 deletion reduced thermotolerance in the 21A strain background (Figure S2 online).

Since 21A with either mkt121A or MKT1BY4742 showed the same growth at 40.7°C and since BY4742 showed the same growth at 40.7°C as BY4742 mkt121A, the MKT121A allele behaves as a loss of function allele for thermotolerance when assayed under our conditions and in our haploid strain backgrounds (Figure S2 online).

In a previous QTL mapping study of thermotolerance with a clinical isolate of S. cerevisiae and the lab strain S288c, the MKT1 allele of the clinical isolate was also identified as a causative gene [6] and in a follow-up study, out of two polymorphisms in Mkt1, D30G and the conservative substitution K453R, the D30G mutation was identified as the causative mutation [27]. Sanger sequencing of MKT121A confirmed that Mkt1-21A has the same mutations. END3 and RHO2, which are located close to MKT1 in the same QTL, were also reported to have an allele-specific contribution to thermostolerance [6]. However, in the current experimental setup, the RHO2 allelics from our two genetic backgrounds did not produce a difference in thermostolerance, while for END3 there may be a slight difference (Figure 3B).

Identification of the causative gene in QTL3

QTL3 is linked to the genome of the inferior parent strain, indicating that BY4742 contains a superior genetic element for thermostolerance in this region. We fine-mapped QTL3 by scoring seven selected SNPs in 62 thermostolerant segregants individually. This reduced the locus to 40,000 bp (Figure 4A). Detailed analysis of the 21A sequence in this region revealed 13 genes and putative ORFs with at least one non-synonymous mutation (Figure 4A).

Table 1. List of QTLs identified in the mapping with the original parents.

| QTL     | Location         | Total number of thermotolerant segregants used | Association to superior parent strain | FDR p-value |
|---------|------------------|-----------------------------------------------|--------------------------------------|-------------|
| QTL1    | 435069–475213 on chromosome XIV | 46                                            | 100%                                  | 5.16e-13    |
| QTL2    | 540838–560167 on chromosome II | 62                                            | 74.20%                                | 1.60e-3     |
| QTL3    | 949927–999889 on chromosome IV | 62                                            | 29.03%                                | 1.18e-2     |

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We also tested growth at high temperature of strains containing a heterozygous deletion of either the complete FRAGMENT1 or only the PRP42 gene together on the same plate. We found the growth at 41°C to be similar whether the complete FRAGMENT1 or only the PRP42 gene from either BY4742 or from 21A was deleted (Figure S4 online). This suggests that PRP42 was likely the only causative gene in FRAGMENT1 and thus also seems to exclude the other genes without non-synonymous mutation in their ORF as possible causative gene. As an additional control, we also performed RHA with the seven genes of Fragment 2 with a non-synonymous mutation in their ORF and we did not find any difference between the alleles from the two parent strains in conferring thermotolerance (data not shown).

PRP42^21A has eleven mutations compared to PRP42^BY4742, with three of them being non-synonymous and the other eight synonymous (Table S2 online). The three polymorphisms in Prp42, H296Y, F467S, and E526Q, are non-conservative substitutions, but it is difficult to predict a possible effect on the function or structure of the protein. They are located in domains without strong conservation (data not shown). Since no mutation was present in the promoter and terminator region, the difference in thermotolerance conferred by the two PRP42 alleles is likely due to the change in protein sequence and thus in functionality. We have investigated the presence of these mutations in 22 other yeast strains, isolated from various sources, and of which the whole genome has been sequenced (Table S2 online), and found that among the three non-synonymous mutations, C886T is unique to 21A, whereas the other two mutations (C1400T and C1576G) are present in all other strains except in the lab strains S288c, CEN.PK113-7D and W303. If we assume that the inferior PRP42 allele is rare (like the inferior MKT1 allele in S288c), then C886T is the best candidate for the causative mutation. On the other hand, we cannot exclude that C886T is only one of the causative mutations, that it requires interaction with one or more of the other mutations or that a combination of the other SNPs is causative for the phenotype.

Construction and phenotyping of the downgraded parent strains

We next constructed two downgraded parent strains each with their own superior allele replaced by the inferior allele of the other parent: 21ADG: 21A mkt1D::MKT1BY4742 and BY4742DG: BY4742 prp42D::PRP4221A. Growth at 41°C of 21A DG was reduced compared to 21A, confirming the importance of MKT1^21A for high thermotolerance in 21A (Figure 5A). At 41°C, BY4742 and also BY4742DG are not able to grow (Figure 5A). Hence, we reduced the temperature to 40.7°C, which allowed to demonstrate reduced growth of BY4742DG compared to BY4742 (Figure 5B). Also at 41°C, we could demonstrate the beneficial effect of PRP42^BY4742 compared to PRP42^21A by comparing growth of the 21A DG/21A/BY4742 and 21A DG/BY4742DG hybrid strains (Figure 5A). The availability of the four hybrid diploid strains also allowed us to demonstrate that in this background the effect of the MKT1 and PRP42 genes on thermotolerance is independent. The hybrid diploids, 21A mkt1D/21A/BY4742 and 21A prp42D/BY4742, each with replacement of one superior allele, both showed reduced growth at 41°C compared to the original hybrid of the parent strains, 21A/BY4742, while the hybrid of the two downgraded parent strains, 21A mkt1D/21A prp42D/BY4742DG, in which both superior alleles are replaced, showed further reduced growth (Figure 5A). (In this figure all strain pairs were put on the same plate.)

Figure 3. Dissection of QTL1 to identify the causative gene. (A) Fine-mapping of QTL1 by scoring selected SNPs in the individual thermotolerant segregants. Eight SNPs spanning between 400,000 bp and 550,000 bp on chromosome XIV were scored by PCR in 46 thermotolerant segregants and both SNP variant frequency and FDR p-value were calculated. A 60,000 bp region between SNP2 and 5 showed the strongest linkage. It contained 33 genes and putative ORFs as indicated using the annotations in SGD. The genes containing at least one non-synonymous mutation within the ORF are indicated with an asterisk. (B) Identification of the causative gene MKT1 in QTL1. RHA results for MKT1, RH02 and END3 in the central region of QTL1 are shown. The strain pairs for the same genes were always spotted on the same plate. The results for the original hybrid diploid 21A/BY4742 and the MKT1 reciprocal deletion strains were also from the same plate. doi:10.1371/journal.pgen.1003693.g003
Isolation and phenotyping of segregants from the downgraded parent strains

Figure 5 shows that both at 41°C and 40.7°C, the two downgraded parent strains, 21ADG and BY4742DG, still show a strong difference in thermotolerance. We sporulated the 21ADG/BY4742DG diploid strain and phenotyped 2464 segregants for thermotolerance. Examples are shown in Figure 5B. The segregants showed a range of thermodiversity and also transgressive segregation [28], since some of the segregants showed poorer thermodiversity than the inferior BY4742DG parent (e.g. segregant 9 in Figure 5B) while others showed better thermodiversity than the superior 21ADG parent (e.g. segregant 8 in Figure 5B). This suggests the presence of additional QTLs and causative genes influencing thermodiversity.

Identification of new QTLs with segregants from the downgraded parents

From the 2464 segregants derived from the diploid 21ADG/BY4742DG, we selected 58 thermodiverse segregants that grew at 40.7°C at least as well as the 21ADG superior parent strain, and repeated the pooled-segregant whole-genome analysis. We have used the same set of SNPs as generated in the previous sequencing of the 21A parent strain compared to S288c, for the mapping of QTLs linked to thermodiversity. A total of ten regions have a two-sided p-value low enough for significance (Figure 2). Interestingly, two regions can be discerned with a clear difference between the original and downgraded pool (Figure 2, Table S1 online). The previous peak indicating linkage of one or more causative elements in the region between about 400,000 bp and 600,000 bp on chromosome XIV with the superior parent 21A (QTL1) has shifted to a more upstream position in the mapping with the 21ADG downgraded superior parent (QTL4). In the region between 600,000 bp and 800,000 bp on chromosome XII, there is a new conspicuous peak, indicating linkage with the 21ADG superior parent (QTL5). We confirmed the statistical significance of these two new QTLs by scoring selected SNPs in the individual thermodiverse segregants and performing a binomial exact test (Table 2). For the remaining seven regions, the SNPs showed about 50% variant frequency in the unselected pool (Figure S5).
Figure 5. Thermotolerance of the downgraded parent strains and their segregants. (A) Growth at 41°C of the original parent strains, 21A and BY4742, the downgraded parent strains, 21ADG and BY4742DG, and hybrid diploids in the four combinations. All strains were spotted on the same plate. (B) Growth at 40.7°C of the original parent strains, 21A and BY4742, the downgraded parent strains, 21ADG and BY4742DG, and ten segregants from the hybrid 21ADG/BY4742DG. The strain pairs for each gene were always spotted on the same plate.

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online). This suggests that the putative weak linkage from these regions is not caused by allelic incompatibilities. In addition, the significant association of the causative element(s) in QTL3 with the inferior parent (71% of the segregants had the genotype of the inferior parent, as determined by individual segregant genotyping) observed in the first mapping was completely abolished in the second mapping (52% of the segregants had the genotype of the inferior parent), which reaffirms that PRP42 is the only causative gene in this locus.

**Identification of causative genes in the new QTL4 and QTL5**

In a previous QTL mapping study of thermotolerance [22], the authors identified the NCS2 allele of a clinical isolate as a superior allele compared to the inferior allele from the S288c control strain. Since NCS2 is located in the central region of QTL4 and in the central region of QTL4 and the NCS2<sup>BY4742</sup> allele contains the same mutation (A212T) as identified in the previous study, we have tested whether NCS2<sup>BY4742</sup> is also a causative allele in our genetic background. For that purpose, we performed RHA for NCS2 using a hybrid diploid strain constructed from the two downgraded parent strains. We found that the NCS2<sup>BY4742</sup> allele supported higher thermotolerance compared to the NCS2<sup>S221A</sup> allele, indicating that also in our genetic background the NCS2 allele from the superior strain acted as a causative gene (which does not preclude the presence of other causative genes). Deletion of the inferior NCS2<sup>BY4742</sup> allele in the hybrid diploid strain also caused a conspicuous drop in thermotolerance (Figure S6 online).

Fine-mapping of QTL5 by scoring six selected SNPs individually in all 58 thermotolerant segregants enabled us to reduce the size of the QTL from 150,000 bp to 40,000 bp (Figure 6A). We then divided this region into three fragments and performed bulk RHA with each fragment in the 21A<sup>DG</sup>/BY4742<sup>DG</sup> diploid strain (Figure 6A). (The fragments had an overlap of one gene.) Evaluation of thermotolerance with the pairs of reciprocally deleted hemizygous strains revealed that FRAGMENT<sub>21ADG/BY4742DG</sub> and FRAGMENT<sub>21A/BY4742</sub> conferred higher thermotolerance than the corresponding fragments from the inferior BY4742<sup>DG</sup> parent. For FRAGMENT<sub>3</sub> there was no difference (Figure 6B). We then performed RHA with all individual genes of Fragments 1 and 2 containing non-synonymous mutations in their ORF (as indicated in Figure 6A). However, for none of the genes tested there was a different effect on thermotolerance of the two alleles (data not shown). We then applied RHA to the remaining genes in FRAGMENT<sub>2</sub> and found that the SMD2<sup>21A</sup> allele conferred higher thermotolerance compared to the SMD2<sup>BY4742</sup> allele (Figure 6B). Hence, it apparently acted as a causative allele in both FRAGMENT<sub>1</sub> and FRAGMENT<sub>2</sub>, since it was the only gene present in the overlap between the two fragments. The observation that replacement of FRAGMENT<sub>1</sub> with FRAGMENT<sub>1</sub> caused a similar reduction in thermotolerance compared to the replacement of FRAGMENT<sub>2</sub> with FRAGMENT<sub>2</sub> is consistent with SMD2 being the only causative gene in QTL5.

We confirmed by Sanger sequencing that SMD2<sup>21A</sup> only displayed SNPs in the promoter and terminator region as compared to SMD2<sup>BY4742</sup> (data not shown). Hence, a difference in expression level may be responsible for the difference in thermotolerance. We have compared SMD2 transcription levels in different strains and with incubation at different temperatures. We found a higher level of SMD2 expression in 21A compared to BY4742 in cells growing exponentially in liquid cultures (YPD at 30°C) and also 21A<sup>DG</sup> showed a higher level of SMD2 expression under these conditions than BY4742<sup>DG</sup> (Figure 6C). The difference in SMD2 expression level is also clear for the 21A/ BY4742 RHA pairs, but there is no significant difference for the 21A<sup>DG</sup>/BY4742<sup>DG</sup> RHA pairs (Table S3 online). This indicates that the mechanism of SMD2 in influencing thermotolerance cannot be solely due to differences in its transcript level, and other mechanisms such as post-transcriptional regulation may play a role.

**Detection of an allele-specific epistatic interaction between PRP42 and SMD2**

In the cross with the original parents, the QTL5 region did not show any indication of linkage to the genome of the superior parent strain 21A, with 37 out of 58 thermotolerant segregants of 21A/BY4742 having the SMD2<sup>21A</sup> allele (confirmed by genotyping the individual segregants, data not shown). We have also applied RHA for SMD2 in the original 21A/BY4742 hybrid. Interestingly, we could not detect any difference in thermotolerance at the two temperatures tested (40.7°C and 41°C) (Figure 7A). Knowing that 21A<sup>DG</sup>/BY4742<sup>DG</sup> lacks only two superior alleles as compared to 21A/BY4742 and both PRP42 and SMD2 encode proteins forming the same spliceosomal complex, we constructed double hetero-allelic mutations for PRP42 and SMD2 in the 21A/BY4742 background, and evaluated thermotolerance of the strains. In the hybrid with the inferior PRP42 allele, the superior SMD2 allele caused higher thermotolerance compared to the inferior SMD2 allele, whereas in the hybrids containing the superior PRP42 allele, the two SMD2 alleles did not influence thermotolerance differently (Figure 7B). The identification of SMD2 as a causative gene for thermotolerance indicates that our new approach of mapping with the downgraded parent strains is able to reveal minor loci and causative genes that escape detection in QTL mapping with the original parents, in this specific case because of epistatic interaction.

**Expressing PRP42<sup>BY4742</sup> in 21A does not enhance its thermotolerance**

We expressed the two PRP42 alleles from a centromeric plasmid in the parent 21A strain (Figure S7A online) and in the 21A<sup>ped2A</sup> strain (Figure S7B online). In both cases, there was no difference in thermotolerance between the strains. On the other hand,
hand, comparison of the thermotolerance of strain 21A and that of the two heterozygous RHA strains showed that the RHA strain expressing the 21A allele had clearly lower thermotolerance than the other two strains (Figure S7C online). The thermotolerance of the heterozygous RHA strain expressing the superior PRP42 allele from BY4742 was not higher than that of the 21A strain. These results show that the BY4742 allele of PRP42 is not able to enhance the thermotolerance level of the 21A strain further, apparently indicating that other factors become limiting for thermotolerance. One such other factor may be SMD2. In the 21A strain it is present for 100% in the superior form, while in the heterozygous RHA strain, it is only present for 50% in the superior form. Hence, a dosage effect of SMD2 may possibly be limiting for thermotolerance in the heterozygous RHA strain expressing the superior PRP42 allele from BY4742. The difference in ploidy or in the genetic constitution between the haploid 21A strain and the diploid RHA hybrid strains may also play a role, although this seems to be contradicted by the fact that we mapped the superior PRP42 allele using haploid segregants of the superior and inferior parents. Also in the study of Sinha et al. [27], replacement of the inferior allele of MKT1 with the superior allele in the S288c strain did not cause the expected improvement in thermotolerance.

Discussion

Identification of QTLs with minor effects on complex traits remains a difficult issue in quantitative genetics [29]. Major approaches used up to now have been fixing of major QTLs in a
single parent and repeating the QTL mapping procedure either with backcrosses or regular crosses between the parents [22,23,30], the use of very high numbers of segregants [24], more stringent phenotyping to enhance the detectability of the minor QTLs [10] or genotyping and phenotyping single segregants [21].

In this study, we have extended the approach of fixing major QTLs to mapping by pooled-segregant whole-genome sequence analysis. In addition, we fixed a major QTL in each parent strain to create a downgraded superior and a downgraded inferior parent strain. The benefit of downgrading both parents, especially in pooled-segregant mapping, is that it keeps a large phenotypic difference between the parental strains. This makes the isolation of a sufficient number of segregants with extreme phenotype easier or at least makes the evaluation of their phenotype in comparison with that of the superior parent easier. In addition, it may enhance the chances that the minor QTLs identified are truly relevant for the phenotypic difference between the original parents and not generated in some unrelated manner.

Our approach is based on the observation that the causative genetic element(s) in some QTLs is(are) linked to the inferior rather than to the superior parent. This is likely due to the fact that genetic mapping in yeast is performed with haploid strains derived from natural or industrial diploid strains that generally harbor a single copy of many recessive alleles. As a result of the presence of negative, recessive mutations, positively acting QTLs and causative genes will be identified that are linked to the inferior rather than the superior parent. This has also been observed in several previous mapping studies [10,18,24]. It indicates that linkage of QTLs to the inferior parent is not an uncommon phenomenon and, moreover, may significantly increase when the influence of major QTLs is weakened or when genetic linkage in the genome is reduced.

Identification of the causative gene in QTL1, linked to the superior parent, and in QTL3, linked to the inferior parent, allowed us to construct both a downgraded, superior and a downgraded, inferior parent strain using targeted allele replacement. Repeating the genetic mapping with the downgraded parent strains successfully revealed new minor QTLs and thus established the effectiveness of this approach. Moreover, we validated the new QTLs 4 and 5 by identifying the causative genes. QTL4 contained a causative gene previously identified for high thermotolerance in another yeast background [22], further underscoring the effectiveness of this approach. Interestingly, our identification in the cross with the downgraded parent strains of new QTLs linked to both superior and inferior parent, allows in principle to construct...
functions of these genes underscores our limited understanding of overlap with the regions in which these genes are located, except in natural yeast strains with an allele-specific contribution to NCS2 could subsequently confirm upstream position. In the new QTL, which was called QTL4, we allowing identification of the remaining causative gene(s). This segregants of the downgraded parents. On the other hand, if QTL3, for which there was no linkage anymore with the completely in the second cross. In our case, this happened with only causative gene in their QTL, this QTL should disappear or even within the previously identified QTL. If the superior alleles that have been replaced in the downgraded parent strains were the only causative gene in their QTL, this QTL should disappear completely in the second cross. In our case, this happened with QTL3, for which there was no linkage anymore with the segregants of the downgraded parents. On the other hand, if other causative genes exist within the QTL in addition to the fixed gene, the QTL will likely remain present in the second mapping, allowing identification of the remaining causative gene(s). This happened in our case with QTL1, which shifted to a slightly more upstream position. In the new QTL, which was called QTL4, we could subsequently confirm NCS2 as the causative gene. The presence of multiple causative genes located close to each other within a single QTL has been found before [6,10,22]. To resolve closely located QTLs in the first cross an impractical number of F1 segregants is easily required [31]. Recently, multiple, random inbreeding with all F1 segregants was used to enhance recombination between the genomes of the parents and thus reduce linkage in the genome. This resulted in a higher resolution of genetic mapping, facilitating detection of closely located minor QTLs and also strongly reduced the number of candidate genes in the centre of the QTL [24].

The appearance of new minor QTLs in the second mapping, with QTL5 and its causative gene SMD2 as a striking example, raises the question why these QTLs were not detected in the first mapping. One plausible explanation is interaction between causative genes from different QTLs, which has been identified by Lorenz et al. [23]. In our study we identified a negative interaction between the SMD2 and PRP42 alleles, which can explain the absence of QTL5 in the first mapping. In the latter, the presence of the superior PRP42 allele in the selected heterozygous segregants could compensate for the presence of an inferior SMD2 allele. In the second mapping, after removal of the superior PRP42 allele, the effect of the superior SMD2 allele now apparently became more significant, causing a higher chance for this allele to be present in the thermotolerant segregants.

Thermotolerance of growth, which is the ability to grow at elevated temperatures, has been a favourite trait in quantitative genetics with yeast [6,22,24,27,32,33]. It is easily scored on solid nutrient plates, it is highly relevant for several industrial applications with yeast and is a typical characteristic of clinical isolates of S. cerevisiae. To date, several genes have been identified in natural yeast strains with an allele-specific contribution to thermotolerance. The QTLs identified in our study did not overlap with the regions in which these genes are located, except for QTL1 (MKT1) and QTL4 (NCS2). The diverse biological functions of these genes underscores our limited understanding of this phenotype, since apparently none of these genes has a function that can be directly linked in a known mechanistic manner to sustaining high thermotolerance.

In this study, we have identified PRP42^{BY74} and SMD2^{214} as two novel and naturally-occurring superior alleles for high thermotolerance. Yeast Prp42 was identified as an essential protein for U1 small nuclear ribonucleoprotein (snRNP) biogenesis, which has a high similarity to Prp39 [34]. SMD2 encodes a core protein Sm D2 that is part of the spliceosomal U1, U2, U4, and U5 snRNPs [35]. These snRNPs function in pre-mRNA splicing by recognizing short conserved sequences from 5' to 3' at the exon-intron junctions and assemble into active splicosomes [36]. Interestingly, the related function of these two genes suggests an important role for RNA processing in growth at high temperature. Further analysis revealed an allele-specific interaction between PRP42 and SMD2. This is consistent with the previous evidence for direct interaction between the human homologues of these gene products as revealed by crystal structure determination of human spliceosomal U1 snRNP [37].

The MKT1 gene has been found as a causative gene in several QTL mapping studies with various phenotypes and using diverse genetic backgrounds, but always with the S288c/BY background for the control parent [10,13,30,38]. MKT1 appears to control gene expression at a post-transcriptional step [39], which may explain why its deficiency produces effects on such a diversity of phenotypes.

To allow faster identification of causative genes in the mapped QTLs, we have applied bulk RHA, which evaluates multiple adjacent genes simultaneously. The successful identification of causative genes (PRP42 and SMD2) using this approach confirms the effectiveness of this method. A possible advantage of this strategy over RHA with single alleles is that it can take into account genetic interactions [19] between the genes in the deleted region. If two closely located genes can compensate for each other, bulk RHA may detect their effect as opposed to single gene RHA. Another advantage of bulk RHA is its high efficiency, especially in cases where QTLs cannot be reduced to a small size with only few genes in the centre because of a limited number of segregants available for fine-mapping. In general, this will be the case with phenotypes that require a high workload for scoring. In our experience, with bulk RHA one can easily evaluate a region with a size of 20 kb, which encompasses on average between 6 and 12 genes in yeast. On the other hand, bulk RHA carries possible pitfalls. When a region used for bulk deletion carries both positively acting and negatively acting genes, as was found in previous studies [6,10], simultaneous deletion of both can result in the absence of any phenotypic effect. Hence, a negative result with bulk RHA does not necessarily imply the absence of causative genes.

Conclusions

In this paper we have shown that identification of new minor QTLs involved in complex traits can be successfully accomplished by crossing parent strains that have both been downgraded for a single QTL. Using this approach we have identified new QTLs and new causative genes, revealing an important role for RNA processing in high thermotolerance. This method has the advantage of maintaining all relevant genetic diversity and enough phenotypic difference between the two parent strains and thus significantly increases the chances of identifying minor QTLs. In principle, successive rounds of minor QTL mapping could be performed in this way by sequentially downgrading the two parent strains further, making use each time of a causative gene identified
in a QTL linked to the superior parent and in a QTL linked to the inferior parent.

**Materials and Methods**

**Yeast strains, growth conditions and sporulation**

The following yeast strains were used: prototrophic and heterothallic diploid strain MUCL28177, which was isolated from orange juice in the region of Strombeck-Bever, Belgium, its haploid segregant MUCL28177-21A, referred to as 21A, and BY4742 \([\text{Mat}^a \text{his}3 \Delta 1 \text{leu}2 \Delta 0 \text{ura}3 \Delta 0 \text{met}10 \Delta 0]\) [40]. Yeast cells were grown in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) bacteriological peptone, and 2% (w/v) glucose. 1.5% (w/v) Bacto agar was used to make solid nutrient plates. Transformants were grown on YPD agar plates containing 200 μg/ml geneticin. Mating, sporulation and isolation of haploid segregants were done using standard protocols [41].

**Phenotyping**

Strains were inoculated in liquid YPD and grown in a shaking incubator at 30°C overnight. The next day the cells were transferred to fresh liquid YPD at an OD₆₀₀ of 1 and grown for 2 to 4 h to enter exponential phase. The cell cultures were then diluted to an OD₆₀₀ of 0.5 and 5 μl of a fourfold dilution range was spotted on YPD agar plates, which were incubated at different temperatures. Growth was scored after two days incubation for all conditions. All spot tests were repeated at least once, starting with freshly inoculated cultures. Repetitions of the thermotolerance assays may show slight differences in growth intensity. Hence, the strains to be tested were always spotted together with the relevant controls on the same plate.

**Pooled-segregant whole-genome sequence analysis and determination of SNP variant frequency**

**Whole-genome sequencing.** For each genetic mapping experiment, 58 thermotolerant segregants were grown separately in 50 ml liquid YPD cultures at 30°C for three days. Cell dry weight was measured for each culture and the cultures were pooled based on the same dry weight. Genomic samples of the pooled culture, together with that of 21A were isolated with standard methods [42]. At least 5 μg of each DNA sample was provided to GATC Biotech AG or BGI for sequencing. Paired-end short reads of 100 bp were generated. Sequence alignment was performed using SeqMan NGen. Assembly and mapping were done with DNAstar Lasergene.

**Filtering.** SNPs were selected for high quality, based on filtering for sufficient coverage (≥20 times) and ratio (≥80%) [10,25]. The coverage of at least 20 times was based on previous findings that a 20-fold sequencing coverage is sufficient to compensate for errors by the number of correct reads [43]. The ratio of at least 80% was chosen based on the plots of the SNPs between the two parent strains, as described previously [10,25].

**Statistical model.** Swinnen et al. [10] and Claesen et al. [25] developed an additive logistic regression model for a joint analysis of bulk sequencing data from different pools. They proposed to use simultaneous confidence bands to test for (a) deviations from random segregation (SNP frequency of 50%) or (b) differences between pools along the chromosome, while accounting for multiple testing. In this contribution, we extend the simulation-based inference approach of Claesen et al. [25] and provide adjusted p-values that account for multiple testing. The confidence bands and p-values in the manuscript are based on one million Monte Carlo simulations. Details on the procedure can be found in Supplementary Information: Supplementary Methods.

**Contrast between pools.** We also sequenced a pool of unselected segregants, which is referred to as pool 0 and for which random segregation can be expected. Figure S5 shows patterns in the SNP frequency profile of the unselected pool, which remind of wave effects found in copy number variation profiling [44]. A similar approach to correct for wave patterns has been adopted for bulk segregant sequencing: instead of inferring on deviations from the SNP frequency of 50% (log odds = 0), log odds ratio’s between the selected pools and the unselected pool are assessed.

**Testing against a biological threshold.** Testing if the true log odds ratio between pool q and the unselected pool 0 is different from zero results in statistical significance, but cannot assure that the detected differences are large enough to be biologically meaningful. Following McCarthy and Smyth [45], we test relative to a biological relevant threshold δ for ensuring both statistical significance and biological relevance. The threshold is chosen at δ = 0.4088. This is equivalent to testing if the odds ratio of pool q and pool 0 is outside the interval [2/3,3/2], e.g. it corresponds to testing if the SNP frequency for pool q is outside [40%, 60%] when the SNP frequency of pool 0 equals 50%.

**SNP scoring in individual segregants**

SNPs were scored in individual segregants by PCR. At a given chromosomal location, two SNPs spacing between 500 and 1,500 bp were chosen for the design of specific primers. For a given SNP, two primers either in the forward or reverse direction, were designed with one mismatch at their 3’ ends. First, a gradient PCR was applied using genomic samples of 21A and BY4742 as templates, with each template tested with two primer combinations (primer pair based on the sequence of BY4742 and primer pair based on the sequence of 21A). The annealing temperature at which the best distinguishing power was obtained with the two parents was used for scoring of the SNPs in the individual segregants.

**Statistical analysis.** The SNP data in the individual segregants have been analysed using the binomial exact test. The p-values have been adjusted for multiple testing under dependency using the Benjamini Yekutieli False Discovery Rate (FDR) method [26].

**Reciprocal hemizygosity analysis**

All the ORFs of non-essential genes in the centre of the QTL were deleted separately in both 21A and BY4742. PCR-mediated gene disruption was used [46]. Plasmid pFA6a was used as a template to amplify a linear DNA fragment containing the kanMX4 cassette [47], with 50 bp homologous sequences for the target regions at both ends. Transformants growing on YPD geneticin plates were verified by PCR with several combinations of internal and external primers. The verified haploid deletion strains were subsequently crossed with the matching wild type haploid to generate the hybrid diploids. For RHA with essential genes and fragments containing multiple genes, transformation was performed directly in the hybrid diploid. External SNPs primer pairs together with internal primers within the kanMX4 cassette were used in different combinations to determine in which parent the allele or the fragment had been deleted. For each heterozygous deletion hybrid, at least two isogenic strains were made and evaluated for thermotolerance. The growth of strains in the RHA test should always be compared within the strain pairs and not between the strain pairs, since the loss of one copy of a gene can cause an effect on the growth of the strains under non-restrictive conditions or even under restrictive conditions if the gene is important for the phenotype and because of the variability between different thermotolerance assays.
Allele replacement

The replacement of *MKT1* with *MKT1BY4742* in 21A was performed by a two step transformation. For the first transformation, a linear DNA fragment containing the *AMD1* gene from *Zygosaccharomyces rouxii* flanked by 50 bp sequences that are homologous to the two sides of the *MKT1* ORF was amplified from plasmid pFA6a-AMD1-MX6 [46] by PCR, and transformed into 21A. Transformants were grown on YCB (Yeast Carbon Base 1.17%, phosphate buffer 3%, Bacto agar 2%) plates containing 10 mM acetamide. Single colonies were checked for the correct replacement with the use of external primers. For the second transformation, colonies were transformed with a linear DNA fragment containing the *MKT1BY4742* ORF, together with ~100 bp downstream and upstream. Transformants were grown on YNB galactose (0.17 Yeast Nitrogen Base w/o amino acids and ammonium sulfate, 1.5% Difco agar, 0.01% galactose, pH 6.5) containing 100 mM fluoroacetamide. Colonies were first checked for the presence of *MKT1* by PCR, and then confirmed by DNA sequencing.

The replacement of *PRP42BY4742* with *PRP4221A* in BY4742 was performed in a two step transformation. For the first transformation, a *URA3* gene was inserted ~50 bp downstream of the *PRP42* ORF in BY4742. Colonies growing on −URA plates were confirmed to have a correct insertion by PCR. For the second transformation, a linear DNA fragment containing the ORF of *PRP4221A* together with ~400 bp downstream and upstream was transformed into the previous colonies, and the transformants were grown on 5-FOA plates. Colonies were first checked for the right DNA polymorphism by SNP primer pairs, and then confirmed by DNA sequencing.

Data access

All sequence data have been deposited in the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) and can be accessed with account number SRA058979.

Supporting Information

Figure S1  RHA for the negative candidate genes in QTL1. For none of these candidate genes there was a clear reproducible difference in thermotolerance between the two hybrid diploids expressing a single copy of the two parental alleles. (PDF)

Figure S2  Effect of *MKT1* deletion on thermotolerance. The *MKT1* gene was deleted in the 21A superior parent strain and in the BY4742 inferior parent strain. Deletion of *MKT1* in the 21A background caused the same drop in thermotolerance as introduction of the BY allele of *MKT1*. In the BY4742 background, deletion of *MKT1* did not affect thermotolerance. All strains were spotted on the same plate and incubated at 40.7°C. (PDF)

Figure S3  RHA for the remaining candidate genes within *FRAGMENT1* of QTL3. For none of these candidate genes there was a clear reproducible difference in thermotolerance between the two hybrid diploids expressing a single copy of the two parental alleles. (PDF)

Figure S4  RHA with *FRAGMENT1* and *PRP42* of QTL3. *FRAGMENT1* and *PRP42* from the BY background cause a similar increase in thermotolerance compared to *FRAGMENT1* and *PRP42* from the 21A background, suggesting that *PRP42* is the main causative gene in *FRAGMENT1*. All strains were spotted on the same plate and incubated at 41°C. (PDF)

Figure S5  Plot of the SNP variant frequency against the SNP chromosomal position for the pool of unselected segregants. The genomic DNA of the pool of 58 unselected segregants from the hybrid strain 21A<sup>DCG</sup>/BY4742<sup>DCG</sup> was sequenced and analyzed in the same way as for the selected segregants. The top-panel represents the SNP variant frequency (small gray circles) along with the smoothed SNP frequency profile (black line) using an additive logistic regression model. In the middle panel the log odds of the SNP variant frequency is plotted for pool 0 along with simultaneous 95% confidence bands (gray regions). The bottom panel shows 2-sided p-values along the chromosome that are corrected for multiple testing. The SNP variant frequency only shows random variation throughout the genome. (PDF)

Figure S6  RHA for the candidate causative gene *NCS2* in the new QTL4 identified with the downgraded parents. *NCS2<sup>21A</sup>* conferred higher thermotolerance than *NCS2<sup>BY4742</sup>*. Deletion of *NCS2BY4742* in 21A<sup>DCG</sup>/BY4742<sup>DCG</sup> also reduced thermotolerance, indicating that *NCS2<sup>BY4742</sup>* is not a non-functional allele. (PDF)

Figure S7  Expression of *PRP42* alleles in 21A. (A) *PRP42* alleles were expressed from a centromeric plasmid in 21A. (B) *PRP42* alleles were expressed from a centromeric plasmid in 21A *prp42*. (C) Growth of 21A and the RHA pair for *PRP42* on the same plate. (PDF)

Table S1  List of putative QTLs for both original and downgraded parents. (DOCX)

Table S2  Presence of the *PRP42<sup>21A</sup>* ORF SNPs in other yeast strains with various origins. (DOCX)

Table S3  SMD2 expression analysis. (DOCX)

Text S1  Supplementary methods. (PDF)

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Author Contributions

Conceived and designed the experiments: YY MRFM AT KS FD JMT. Performed the experiments: YY EE. Analyzed the data: YY MRFM LC AT KS FD JMT. Wrote the paper: YY LC JMT.
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