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Targeted-deletion hemizygote construction by homologous recombination

A given targeted hemizygote for each RH-seq hit gene except *TAF2* was generated in the *S. cerevisiae* DBVPG1373 x *S. paradoxus* Z1 hybrid (JR507) by knocking out the allele of the gene from one species via homologous recombination with KANMX as described[37] with 70 base pairs of homology on the 5' and 3' ends of the cassette; checking was via diagnostic PCR. Two or more independent transformants were isolated and phenotyped for each hemizygote genotype (Supplementary Table 2).

Construction of allele replacement and targeted hemizgyote strains with Cas9

At each RH-seq hit gene, we constructed strains in wild-type homozygous diploid *S. cerevisiae* DBVPG1373 in which both copies of the endogenous allele were replaced by the allele from an *S. paradoxus* isolate (Z1 for Figure 3 and Supplementary Figures 8 and 9, and other strains as indicated for Supplementary Figure 10), and likewise for replacement of alleles from *S. cerevisiae* (DBVPG1373 in Figure 3 and Supplementary Figures 8 and 9, and other strains as indicated for Supplementary Figure 10) into *S. paradoxus* Z1. We call each such strain an allele-replacement strain, and each was constructed using a dual-guide Cas9 transgenesis method[38] in which a linear PCR fragment from the donor species is incorporated into the recipient genome by homology-directed repair of two chromosomal double-strand breaks induced by Cas9. Briefly, for each allele of each gene, we designed two guide RNAs for double-strand breaks by Cas9: one guide targeted a position ~1000 base pairs 5' to the coding start or at the

3’ end of the closest upstream gene, whichever was closer, and the other guide targeted the region of the coding stop. The precise cut site of each was chosen to contain an NGG immediately downstream of variants between the *S. cerevisiae* and *S. paradoxus* strains, to avoid re-cutting of the donor allele by Cas9 after it had been introduced into the recipient strain. We cloned the two guide RNAs, a KANMX cassette, and the gene encoding the *S. pyogenes* Cas9 protein into a single plasmid as described[38]. The resulting plasmid was propagated in DH5a *E. coli* and miniprepped with a column miniprep kit (Qiagen). Separately, to generate the fragment to be used as the donor for DNA repair after Cas9 cutting, we PCR-amplified the respective region from the donor strain, with primers whose 5’-most 70 base pairs were homologous to the recipient and whose 3’-most 20 base pairs were homologous to the donor, except in the case of transgenesis using *S. cerevisiae* donors other than DBVPG1373 or *S. paradoxus* donors other than Z1 (for Supplementary Figure 10), for which the 3’-most 20 base pairs were homologous to DBVPG1373 or Z1, respectively. The homology region at the 5’ end of the gene ended at most 31 base pairs upstream of the 5’ cut site, and the homology region at the 3’ end of the gene started at most 33 base pairs downstream of the 3’ cut site. The donor fragment product was purified with a column kit (Qiagen) and ethanol-precipitated. We then simultaneously transformed, using the lithium acetate method, the donor fragment and dual-guide Cas9 plasmid into the recipient strain, using donor:acceptor ratios of 0.38:10 to 1:5, with 0.5-26 ug of plasmid. In this transformation, heat shock was for 20-30 minutes at 39-42°C in transformations of *S. cerevisiae* DBVPG1373, and 10-20 minutes at 37-39°C for transformations of *S. paradoxus* Z1. Transformants were plated on YPD+G418 (300 µg/mL) to select for cells that retained the plasmid. From this selection we patched single colonies onto YPD without G418, under the expectation that by the time a lawn came up for each patch, its cells would have lost the Cas9 plasmid. Each such strain was Sanger-sequenced at the junctions of the recipient and donor sequence. Positive patches were streaked to single colonies on YPD plates, and cells from each such colony were used to inoculate a patch on a YPD plate and, separately,

to inoculate a patch on a YPD+G418 plate. Those colonies whose patches grew on the former but not on the latter were inferred to be cured of the plasmid and stored at -80°C. For all genes except *DYN1*, 2-3 such strains from each transformation were retained for thermotolerance assays and underwent Sanger sequencing of the entire locus to determine the exact swapped region (Supplementary Table 2). For *DYN1* allele-replacement in *S. paradoxus* Z1, the Cas9-based strategy yielded a single verified clone in which the *S. paradoxus* Z1 allele of *DYN1* was replaced by that of *S. cerevisiae* DBVPG1373, and likewise for *DYN1* allele-replacement in *S. cerevisiae* DBVPG1373. In each case, we mated the single swap clone to a wild-type of the respective species background, confirmed heterozygosity of the resulting diploid via allele-specific diagnostic PCR at the *DYN1* locus, sporulated, and dissected tetrads, allowing each spore to autodiploidize and grow up as a homozygote; we retained from one such tetrad the two spores that were homozygous at the *DYN1* locus for the swapped allele, as confirmed by sequencing, and stored these at -80°C.

Targeted-deletion hemizygote strains for *TAF2* were generated by knocking out the *S. cerevisiae* or the *S. paradoxus* allele in the interspecific hybrid (JR507) using the above methods for Cas9 cutting and repair, with the following differences. To generate the fragment to be used as the donor for DNA repair after Cas9 cutting, we PCR-amplified the NATMX cassette from pBC713 (a gift from John Dueber, constructed as in [39]) using primers whose 5'-most 70 base pairs were homologous to the recipient and whose 3'-most 20 base pairs were homologous to the cassette. The homology region at the 5' end of the gene ended 22 base pairs outside the 5' cut site (which was upstream of coding start), and the homology region at the 3' end of the gene started 33 base pairs outside the 3' cut site. Positive strains were confirmed by PCR. Two independent transformants were isolated and phenotyped for each genotype (Supplementary Table 2).

Growth assays

Growth measurements of wild-type SGRP strains.

For the growth timecourse of a given wild-type, purebred homozygote isolate from the SGRP collection at 28°C, it was streaked from a -80°C freezer stock onto a YPD agar plate and incubated at 26°C for 3 days. A single colony was inoculated into liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm. This culture was back-diluted into YPD at an OD_{600} of ~0.05 and grown for an additional 5.5 hours at 28°C, 200 rpm, until reaching logarithmic phase. We transferred cells from each such pre-culture, and YPD, to each of 11 replicate wells of a 96-well plate, with volumes sufficient to yield a total volume of 150 µL per well at an OD_{600} of 0.02. The plate was covered with a gas-permeable membrane (Sigma) and incubated with orbital shaking in an M200 plate reader (Tecan, Inc.) at 28°C for 24 hours. For curves in Supplementary Figure 1a, measurements for optical density at 595nm (OD_{595}) were taken every 30 minutes and for each timepoint, the average was taken across replicate wells. To subtract background OD_{595} for the resulting curve, we tabulated the mean of the five lowest values from all datapoints, excluding the first two, and subtracted this value from that of each timepoint, setting any negative value to 0. To smooth the resulting curve, we first replaced each timepoint measurement by its average with those of the timepoints immediately before and after it; then, for any timepoint whose measurement was not greater than or equal to the previous one, we set it to be equal to that previous data point. For Supplementary Figure 1b, the efficiency for a given growth curve (from a single well) was calculated as the difference between the OD_{595} measured at the last four smoothed and averaged data points and that of the first four smoothed and averaged data points. Efficiencies from all of the wells from every *S. cerevisia*e isolate were combined and compared to efficiencies from all of the wells for every *S. paradoxus* isolate in a two-sample two-tailed *t*-test.

For the growth timecourse of a given SGRP strain at 39°C (Figure 1A), we used a large-volume flask growth paradigm to avoid the influence of plate effects on growth measurements at high temperature in the incubated microplate reader, as follows. Each strain was streaked from a -80°C freezer stock onto a YPD agar plate and incubated at 26°C for 3 days. A single colony of a given strain was inoculated into liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm. Each of these cultures was back-diluted into YPD at an OD_{600} of 0.05 and grown for an additional 5.5-7.5 hours at 28°C, shaking at 200 rpm, until reaching logarithmic phase. We transferred cells from each such pre-culture, and YPD, to a glass 250 mL flask at the volumes required to attain an OD_{600} of 0.05 in 100 mL YPD, and incubated it at 39°C with shaking at 200 rpm. OD_{600} readings were taken every ~2 hours for ~18 hours. Figure 1A reports representative data from one of three such independent timecourse experiments. For curve fits, we used the getInitial and SSlogis functions in R to estimate starting values for the parameters of the logistic equation, and the nls function to fit the final parameters.

For efficiency measurements of a given SGRP isolate at 39°C in the large-volume format (Figure 1B), it was streaked from a -80°C freezer stock onto a YPD agar plate and incubated at 26°C for 3 days. Two single colonies of each isolate were each inoculated into liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm to generate two replicate pre-cultures. Each was back-diluted into YPD at an OD_{600} at 600 nm of 0.05 and grown for an additional 5.5 hours at 28°C, shaking at 200 rpm, until reaching logarithmic phase. The two pre-cultures were each again back-diluted into YPD in 1-inch diameter glass tubes with a target OD_{600} of 0.05; the actual OD_{600} of each was measured, after which it was grown at 39°C with shaking at 200rpm for 24 hours, and OD_{600} was measured again. The efficiency for each replicate was calculated as the difference between these final and initial OD_{600} values. The pipeline from inoculation off solid plates through pre-culture, two back-dilutions, and growth at 39°C we refer to as a day's

growth experiment. For each day's experiments, we calculated the average efficiency across the replicates of each isolate $\langle e_{strain} \rangle$. We carried out two days' worth of replicate growth experiments for each isolate. For a given species, we used the complete cohort of measurements of $\langle e_{strain} \rangle$ from all isolates of each species, across all days, as input into a two-sample, two-tailed *t*-test to evaluate whether the suite of e_{strain} values across isolates of *S. cerevisiae* was significantly different from the analogous set of values from *S. paradoxus*.

Testing for heterosis in the DBVPG1373 x Z1 hybrid.

To compare efficiency at 39°C between the DBVPG1373 x Z1 hybrid and its *S. cerevisiae* and *S. paradoxus* parent strains (Supplementary Figure 4), each strain was streaked from a -80°C freezer stock onto a YPD agar plate and incubated at 26°C for 3 days. Two single colonies of each isolate were each inoculated into liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm to generate two replicate pre-cultures. Each was back-diluted into YPD at an OD_{600} at 600 nm of 0.05 and grown for an additional 5.5 hours at 28°C, shaking at 200 rpm, until reaching logarithmic phase. The two pre-cultures were each again back-diluted into YPD in 1-inch diameter glass tubes with a target OD_{600} of 0.05; the actual OD_{600} of each was measured, after which it was grown at 39°C with shaking at 200rpm for 24 hours, and OD_{600} was measured again. The efficiency for each replicate, e_{strain}, was calculated as the difference between these final and initial OD_{600} values. The pipeline from inoculation off solid plates through pre-culture, two back-dilutions, and growth at 39°C we refer to as a day's growth experiment. We carried out two days' worth of replicate growth experiments for each isolate. To evaluate the significance of the difference between the growth of the hybrid and that of the *S. cerevisiae* parent, we used the complete cohort of measurements of e_{strain} from each strain, across all days, as input into a two-sample, two-tailed *t*-test.

Growth measurements of targeted-deletion hemizygotes and allele-replacement strains at 28°C.

For efficiency measurements of a given targeted-deletion hemizygote or allele-replacement strain at 28°C (Supplementary Figures 7, 8 and 10), pre-culture and plate reader assays were as for wild-type SGRP strains (see above), except that 6 or more replicate wells were cultured per strain. 2-3 independently isolated targeted-deletion hemizygotes or allele-replacement strains (Supplementary Table 2) were assayed for each genotype. Each timecourse of targeted-deletion hemizygote or allele-replacement strains also included the wild-type hybrid (JR507) or parent (*S. cerevisiae* DBVPG1373 or *S. paradoxus* Z1), respectively, with pre-culture and replication as above. Efficiency for a given growth curve (from a single well) was calculated as the difference between the OD_{600} measured at the last four smoothed and averaged datapoints and that of the first four smoothed and averaged datapoints, with smoothing and averaging as detailed above. For Supplementary Figure 7, relative efficiency for a given well of a given targeted-deletion hemizygote strain at 28°C was tabulated as its efficiency divided by that of the average of all replicate wells of the wild-type hybrid (JR507) grown in the same experiment. For a given gene, we used the complete cohort of these measurements, from all isogenic hemizygotes, as input into a two-sample, two-tailed *t*-test to evaluate whether the relative efficiency of the strain in which the *S. cerevisiae* allele was knocked out was lower than the analogous quantity from the strain in which the *S. paradoxus* allele was knocked out. In Supplementary Figures 8 and 10, allele-replacement strains for a given gene were analyzed analogously, with the relative efficiency calculated against the respective wild-type parent, and with a one-sample, two-tailed *t*-test to evaluate whether the relative efficiency was significantly different from 1.

Growth measurements of targeted-deletion hemizygotes and allele-replacement strains at 39°C.

For efficiency measurements of a given targeted-deletion hemizygote strain at 39°C in the large-volume format (Figure 2B), each strain was streaked from a -80°C freezer stock onto a YPD agar plate and incubated at 26°C for 3 days. Two single colonies of a given strain were each inoculated into liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm. Each such pre-culture at stationary phase, or a log-phase outgrowth of it (which we used in the case of *DYN1* and *TAF2*: the pre-culture and YPD were added at the volumes required to attain an OD_{600} of 0.05 and grown for an additional 5.5 hours at 28°C, shaking at 200 rpm, until the culture reached logarithmic phase) was used to inoculate YPD in 1-inch diameter glass culture tubes with a target cell density corresponding to an OD_{600} of 0.05. The actual OD_{600} of each was measured, after which it was grown at 39°C with shaking at 200 rpm for 24 hours, and OD_{600} was measured again. The efficiency for each such replicate was then calculated as the difference between the final and initial OD_{600} values. The pipeline from inoculation off solid plates through pre-culture, back-dilution, and growth at 39°C we refer to as a day's growth experiment for a targeted-deletion homozygote. In each such experiment, 2-3 independently isolated targeted-deletion hemizygotes of a given gene in each direction were all assayed on the same day, alongside the wild-type hybrid parent (JR507) with replicate structure and methods as above. From each day's experiments, we calculated the average efficiency across the replicates of the wild-type hybrid $\langle e_{hybrid} \rangle$, and we used this quantity to normalize the efficiency $e_{hemizyg}$ measured for each replicate of each hemizygote strain assayed on that day. Thus, the final observable used for analysis for each replicate on a given day was $e_{hemizyg}/\langle e_{hybrid} \rangle$. We carried out 2-3 days' replicate growth experiments for each gene's hemizygotes. For a given gene, we used the complete cohort of these measurements of $e_{hemizyg}/\langle e_{hybrid} \rangle$, from all days and all isogenic hemizygotes, as input into a two-sample, one-tailed *t*-test to evaluate whether $e_{hemizyg}/\langle e_{hybrid} \rangle$ of the strain in which the *S. cerevisiae* allele was knocked out was lower than the analogous quantity from the strain in which the *S. paradoxus* allele was knocked out. To evaluate the agreement between the effect size of

variation at a given locus in these experiments and the estimate from RH-seq data, we first processed the latter as follows. For a given insert in the RH-seq data set we defined thermotolerance as the ratio between $<a_{insert}>_{total}$ after growth at 39°C and $<a_{insert}>_{total}$ after growth at 28°C. We calculated the average thermotolerance across all inserts passing the cutoffs delineated in Supplementary Figure 5 that reflected hybrid strains harboring a wild-type copy of the allele from *S. paradoxus* Z1 of the focal gene and a transposon insertion in the *S. cerevisiae* DBVPG1373 allele; separately, we calculated the analogous quantity from hybrids with an insertion in the *S. paradoxus* allele. The ratio of these two thermotolerance values we refer to as the effect size for the locus of interest as measured in RH-seq. Next, to quantify the effect size for a given locus from our deletion-based hemizygosity experiment, we tabulated the average $e_{hemizyg}/<e_{hybrid}>$, for all isogenic hybrid strains harboring a wild-type copy of the allele from *S. paradoxus* Z1 of the focal gene and a deletion of the *S. cerevisiae* DBVPG1373 allele; separately, we calculated the analogous quantity from strains in which the *S. paradoxus* allele was knocked out. The ratio between these two averages we consider the effect size from deletion-based hemizygotes. We used these two cohorts of effect size values (from RH-seq and deletion-based hemizygosity experiments) as input into a Pearson correlation calculation across all eight loci.

For growth measurements of a given allele-replacement strain at 39°C in the large-volume format (Figure 3, Supplementary Figures 7, 8 and 10), each strain was streaked from a -80°C freezer stock onto a YPD agar plate and incubated at 26°C for 3 days. 1-2 single colonies of each strain were each inoculated into liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm to generate 1-2 replicate pre-cultures. Each was back-diluted into YPD at an OD_{600} of 0.05 and grown for an additional 5.5 hours at 28°C, shaking at 200 rpm, until reaching logarithmic phase. Each pre-culture were each again back-diluted into YPD in 1-inch diameter glass tubes with a target OD_{600} of 0.05 (for experiments using a single pre-culture, it was now

split into two replicate pre-cultures, each of the same OD_{600}); the actual OD_{600} of each was measured, after which it was grown at 39°C with shaking at 200rpm for 24 hours, and OD_{600} was measured again. The efficiency for each replicate was calculated as the difference between these final and initial OD_{600} values. The pipeline from inoculation off solid plates through pre-culture, two back-dilutions, and growth at 39°C we refer to as a day's growth experiment for an allele-swap strain. In each such experiment, 2-3 independently isolated allele-swap strains targeting a given gene in a given background were all assayed on the same day, alongside the respective wild-type background strain (*S. cerevisiae* DBVPG1373 or *S. paradoxus* Z1) with replicate structure and methods as above. For each day's experiments, we calculated the average efficiency across the replicates of the wild-type parent $\langle e_{parent} \rangle$, and we used this quantity to normalize the efficiency e_{swap} measured for each replicate assayed on that day of each allele-swap strain in the respective background. Thus, the final measurement used for analysis for each replicate on a given day was $e_{swap}/\langle e_{parent} \rangle$. We carried out 2-3 days' worth of replicate growth experiments for each gene's allele-swap strains. For a given gene in a given background, we used the complete cohort of measurements of $e_{swap}/\langle e_{parent} \rangle$ from all days and all allele-swap strains as input into a one-sample, one-tailed *t*-test to evaluate whether $e_{swap}/\langle e_{parent} \rangle$ was significantly different from 1. For swaps of the *S. cerevisiae* allele of a given gene into *S. paradoxus*, we tested whether $e_{swap}/\langle e_{parent} \rangle$ was greater than 1 (*i.e.* that the swap strain grew better at 39°C than did its parent), and for swaps of the *S. paradoxus* allele of a given gene into *S. cerevisiae*, we tested whether $e_{swap}/\langle e_{parent} \rangle$ was less than 1 (*i.e.* that the swap strain grew worse at 39°C than its parent).

Testing survival of wild-type parent strains at 39°C.

To test the survival of heat-treated *S. paradoxus* Z1 and *S. cerevisiae* DBVPG1373 (Supplementary Figure 2), each strain was streaked from -80°C freezer stocks onto YPD agar

plates and incubated at 26°C for 3 days. 1-2 single colonies of each parent were inoculated into liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm to create 1-2 replicate pre-cultures. After 24 hours, we transferred cells from each pre-culture, and YPD, to each of 2-4 tubes at the volumes required to attain an OD_{600} of 0.05 in 11 mL YPD. Two tubes were incubated at 28°C and two at 39°C, all with shaking at 200 rpm for 24 hours. OD_{600} of each was measured; for each culture grown at 28°C, 100 µL of a $1.0x10^{-5}$ serial dilution was plated to YPD, and for cultures grown at 39°C, *S. paradoxus* and *S. cerevisiae* were serially diluted to 10^{-1} and $5.0x10^{-5}$, respectively. Plates were incubated at 26°C for three days until single colonies appeared. Colonies on each plate were counted, from which we tabulated the colony forming units per mL of culture plated, and normalized by the optical density of the original plated culture. The pipeline from pre-culture through treatment and colony counting we refer to as one day's worth of experiments. We used the results of two days' worth of experiments (a total of four for each species and temperature) as input into an ANOVA with species and temperature as factors, and took the *p*-value for the interaction between the factors as the estimate of the significance of the difference between species in the effect of temperature (of the 24-hour liquid culture) on cells' ability to grow into colonies (at the permissive temperature).

Microscopy and quantification

For microscopy of *S. cerevisiae* DBVPG1373 and *S. paradoxus* Z1 (Supplementary Figure 3), each species was streaked from a -80°C freezer stock onto a YPD agar plate and incubated at 26°C for 3 days. Two single colonies of each strain were each inoculated into liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm to generate two replicate pre-cultures. Each was then back-diluted into YPD at an OD_{600} of 0.05; one was grown at 39°C with shaking at 200rpm for 24 hours, and the other was grown at 28°C with shaking at 200rpm for 24 hours.

After the 24 hour growth period, 0.5 OD units of each culture were harvested through centrifugation and incubated in 66.5% ethanol for 1-4 hours at room temperature. Each sample was washed twice with 1X Dulbecco's Phosphate Buffered Saline (DPBS, Gibco), resuspended in 0.5 mL of 1X DPBS, and vortexed for 15 seconds on high. 5 µL of each sample was transferred to an agarose pad made with 1% agarose and YPD. We observed samples on a Zeiss Axio Observer inverted bright-field microscope at 100X magnification. Images were taken using a Hamamatsu ORCA-Flash4.0 digital camera and visualized using ZEN software for image analysis. The exposure of each image was set automatically through ZEN, and brightness was adjusted using the "Min/Max" adjustment for black and white light. The pipeline from inoculation off solid plates through pre-culture, growth at 39°C or 28°C, fixation, and imaging we refer to as a day's experiment. We carried out 2 days' worth of experiments for each species, yielding a total of 17-21 images at each temperature, for each species. In each image, free-floating cells were scored manually as singlets or those with a small, medium, or large bud (for a total of 31-151 scored cells per species and temperature). The proportion of scored cells with large buds was tabulated for each day's experiment for each species and temperature.

Locus effect sizes

Locus effect sizes in Supplementary Figure 9 were computed from the data in Figures 2B and 3 of the main text as follows. For analyses in the hybrid background, for a given locus we calculated $m_{S.par}$, the mean of all replicate measurements of $e_{hemizyg}/\langle e_{hybrid}\rangle$ of hemizygote strains lacking the *S. paradoxus* allele. We took, as independent estimates of the locus effect size (each corresponding to a circle on the respective grey bar in Supplementary Figure 9), each measurement of $e_{hemizyg}/\langle e_{hybrid}\rangle$ of a hemizygote strain lacking the *S. cerevisiae* allele, as

a ratio against $m_{S.par}$. For analyses in the *S. cerevisiae* DBVPG1373 background, for a given locus we used each measurement of $e_{swap}/\langle e_{parent} \rangle$ as an independent estimate of the locus effect size (each corresponding to a circle on the respective orange bar in Supplementary Figure 9). For analyses in the *S. paradoxus* Z1 background, for a given locus we used each measurement of $\langle e_{parent} \rangle / e_{swap}$ as an independent estimate of the locus effect size (each corresponding to a circle on the respective blue bar in Supplementary Figure 9).

Supplementary Methods References

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Supplementary Table 1. *S*tatistical tests and resulting *p*-values used in this study. Each row reports the input into, and results from, and the indicated statistical test conducted in the indicated figure or table. Comparison, the strains interrogated for a difference in survival (Supplementary Figure 4) or growth efficiency (all other experiments) at the indicated temperature.

Supplementary Table 2. Strains used in this study. a, Wild-type diploid strains, including those used as parents of the *S. cerevisiae* x *S. paradoxus* hybrid and of allele-replacement transgenesis; SGRP, the Saccharomyces Genome Resequencing Project, version 2. **b**, Hemizygotes in the *S. cerevisiae* DBVPG1373 x *S. paradoxus* Z1 diploid hybrid constructed by targeted deletion of a given species' allele of the indicated gene with the KanMX or NatMX cassette. ΔscYFG::KanMX/spYFG signifies that the *S. cerevisiae* DBVPG1373 allele of YFG was knocked out and the *S. paradoxus* Z1 allele of YFG is intact; strains with the DBVPG1373 allele intact and the Z1 allele knocked out are represented analogously. **c**, Allele replacement strains in *S. cerevisiae* DBVPG1373 or *S. paradoxus* Z1 diploid homozygote backgrounds. In genotype notes, *e.g.* in an *S. paradoxus* background, ΔYFG(-X to +Y)::scYFG(-Z to +W) indicates that in *S. paradoxus* Z1, bases -X to +Y from gene YFG have been removed and replaced by bases -Z to +W of the allele of YFG from the indicated *S. cerevisiae* strain. Positive coordinates count in the 5' to 3' direction from the start codon (+1 corresponds to the A in the ATG), and negative coordinates count in the 3' to 5' direction from the start codon (-1 corresponds to the base directly 5' of the ATG). In cases where the replacement extended into a region of 100% conservation between species, the position of the last divergent nucleotide is shown.

Supplementary Table 3. Depth of RH-seq library sequencing. Each row reports the number of sequencing reads, before mapping, from an RH-seq library made from the DNA of transposon mutant hemizygotes in the *S. cerevisiae* DBVPG1373 x *S. paradoxus* Z1 diploid hybrid. The last four rows report results from the pool immediately after transposon mutagenesis, mutant clone isolation, pooling, and pre-growth at 28°C (time zero, T0). The first 24 rows report results from selection (bulk culture of the pool) at the indicated temperature after inoculation from the T0 sample.

Supplementary Table 4. Primers used in this study. Each row reports a pair of forward and reverse primers used for polymerase chain reaction during the course of the study. "N" represents a random nucleotide of A, T, C, or G. Primers are reported in the 5' to 3' direction, reading from left to right.

Supplementary Table 5. Abundances of transposon-mutant clones in the interspecific hybrid from RH-seq. Each row reports results of sequencing one transposon insertion in the *S. cerevisiae* DBVPG1373 x *S. paradoxus* Z1 diploid hybrid after selection of the transposon mutant pool, reflecting the abundance in the pool of the respective hemizygote clone harboring the insertion. Allele, the species parent's homolog in which the transposon insertion lay. Chromosome, strand, location, and gene, the fine-scale position of the insertion. Abundance, read counts of the transposon insertion sequenced after selection of the mutant pool at the indicated temperature, normalized for library size and averaged across biological and technical replicates. Transposon insertions not detected in any replicate of the indicated selection were assigned an abundance of 1. CV, coefficient of variation over biological replicates of normalized read counts after selection at the indicated temperature.

Supplementary Table 6. Tests of the impact on thermotolerance of variation at each gene in turn via reciprocal hemizygote analysis of clone abundances from RH-seq. Each row reports the results of reciprocal hemizygote tests of thermotolerance of hemizygote transposon mutants at the indicated gene in the *S. cerevisiae* DBVPG1373 x *S. paradoxus* Z1 diploid hybrid, for a total of 3416 genes. Columns B-F report analyses upon the aggregation at the gene level of transposon mutant abundances (Supplementary Table 5) from all biological replicate experiments, filtered for quality control using all parameters detailed in Supplementary Figure 5. For columns G-L, the analogous calculations used data from only a single biological replicate culture, filtered for quality control using only the read-count and insert cutoffs from Supplementary Figure 5. Columns B-D report results of a two-tailed Mann-Whitney statistical test for a difference in the abundance after growth at 39°C, relative to the abundance after growth at 28°C, of hemizygotes harboring transposon insertions in the two species parents' homologs. The Benjamini-Hochberg method was used to correct for multiple testing. The remaining columns report the log ratio of normalized abundances of a hemizygote harboring a transposon insertion in the indicated species parent's homolog after culture at 39°C and 28°C, as a geometric mean across transposon mutants, from all biological replicates (columns E and F) or, in the case of top gene hits, from one biological replicate culture at 39°C and one at 28°C (columns G- L).

Supplementary Table 1

Figure	Comparison	*p*-value	Number of samples	Type of statistical test
1B	Scer vs Spar, SGRP, 39C	3.78E-11	4 cultures for each strain, 3 strains per species, done on one day	two-sample, two-tailed ttest
2B	ΔscAFG2 vs ΔspAFG2, 39C	3.90E-19	8 cultures ΔscAFG2, 8 cultures ΔspAFG2, each done across 2 days accompanied by a total of 4 WT hybrid cultures for normalization	two-sample, one-tailed ttest
	ΔscAPC1 vs ΔspAPC1, 39C	1.78E-05	8 cultures ΔscAPC1, 8 cultures ΔspAPC1, each done across 2 days accompanied by a total of 4 WT hybrid cultures for normalization	two-sample, one-tailed ttest
	ΔscCEP3 vs ΔspCEP3, 39C	4.30E-06	8 cultures ΔscCEP3, 8 cultures ΔspCEP3, each done across 2 days accompanied by a total of 4 WT hybrid cultures for normalization	two-sample, one-tailed ttest
	ΔscDYN1 vs ΔspDYN1, 39C	1.46E-03	8 cultures ΔscDYN1, 8 cultures ΔspDYN1, each done across 2 days accompanied by a total of 4 WT hybrid cultures for normalization	two-sample, one-tailed ttest
	ΔscESP1 vs ΔspESP1, 39C	2.01E-17	8 cultures ΔscESP1, 8 cultures ΔspESP1, each done across 2 days accompanied by a total of 4 WT hybrid cultures for normalization	two-sample, one-tailed ttest
	ΔscMYO1 vs ΔspMYO1, 39C	3.51E-16	12 cultures ΔscMYO1, 8 cultures ΔspMYO1, each done across 2 days accompanied by a total of 4 WT hybrid cultures for normalization	two-sample, one-tailed ttest
	ΔscSCC2 vs ΔspSCC2, 39C	2.03E-07	12 cultures ΔscSCC2, 8 cultures ΔspSCC2, each done across 2 days accompanied by a total of 4 WT hybrid cultures for normalization	two-sample, one-tailed ttest
	ΔscTAF2 vs ΔspTAF2, 39C	1.21E-03	8 cultures ΔscTAF2, 8 cultures ΔspTAF2, each done across 2 days accompanied by a total of 4 WT hybrid cultures for normalization	two-sample, one-tailed ttest
3A	spAFG2 swap into Sc, 39C	3.08E-10	12 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spAPC1 swap into Sc, 39C	0.0009157	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spCEP3 swap into Sc, 39C	7.42E-07	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spDYN1 swap into Sc, 39C	0.000212896	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spESP1 swap into Sc, 39C	6.68E-16	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spMYO1 swap into Sc, 39C	6.23E-05	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spSCC2 swap into Sc, 39C	0.229286169	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spTAF2 swap into Sc, 39C	3.16E-07	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
3B	scAFG2 swap into Sp, 39C	0.062137599	12 cultures swap, done across 3 days accompanied by a total of 6 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scAPC1 swap into Sp, 39C	2.18E-06	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scCEP3 swap into Sp, 39C	0.100537936	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scDYN1 swap into Sp, 39C	0.0324395	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scESP1 swap into Sp, 39C	0.00043022	12 cultures swap, done across 3 days accompanied by a total of 6 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scMYO1 swap into Sp, 39C	0.035129968	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0

Figure	Comparison	*p*-value	Number of samples	Type of statistical test
	scSCC2 swap into Sp, 39C	4.47E-05	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scTAF2 swap into Sp, 39C	0.000790601	12 cultures swap, done across 3 days accompanied by a total of 6 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
S1B	Scer vs Spar, SGRP, 28C	0.067565011	11 cultures for each strain, 3 strains per species, done on 1 day	two-sample, two-sided ttest
S2	Scer vs Spar, survival	0.000152	4 cultures/plates per species per temperature, done across 2 days	Type 2, two-factor ANOVA
S4	Scer vs hybrid, 39C	0.034666896	4 cultures for each species, each done across 2 days	two-sample, two-tailed ttest
S7	ΔscAFG2 vs ΔspAFG2, 28C	0.00636245	12 cultures ΔscAFG2, 12 cultures ΔspAFG2, each done on 1 day accompanied by a total of 6 WT hybrid cultures for normalization	two-sample, two-tailed ttest
	ΔscAPC1 vs ΔspAPC1, 28C	0.152823509	22 cultures ΔscAPC1, 22 cultures ΔspAPC1, each done on 1 day accompanied by a total of 11 WT hybrid cultures for normalization	two-sample, two-tailed ttest
	ΔscCEP3 vs ΔspCEP3, 28C	0.494015046	22 cultures ΔscCEP3, 22 cultures ΔspCEP3, each done on 1 day accompanied by a total of 11 WT hybrid cultures for normalization	two-sample, two-tailed ttest
	ΔscDYN1 vs ΔspDYN1, 28C	0.018791311	22 cultures ΔscDYN1, 22 cultures ΔspDYN1, each done on 1 day accompanied by a total of 11 WT hybrid cultures for normalization	two-sample, two-tailed ttest
	ΔscESP1 vs ΔspESP1, 28C	9.51E-02	22 cultures ΔscESP1, 22 cultures ΔspESP1, each done on 1 day accompanied by a total of 11 WT hybrid cultures for normalization	two-sample, two-tailed ttest
	ΔscMYO1 vs ΔspMYO1, 28C	0.183956326	36 cultures ΔscMYO1, 24 cultures ΔspMYO1, each done on 1 day accompanied by a total of 12 WT hybrid cultures for normalization	two-sample, two-tailed ttest
	ΔscSCC2 vs ΔspSCC2, 28C	0.985768963	36 cultures ΔscSCC2, 24 cultures ΔspSCC2, each done on 1 day accompanied by a total of 12 WT hybrid cultures for normalization	two-sample, two-tailed ttest
	ΔscTAF2 vs ΔspTAF2, 28C	0.05848207	20 cultures ΔscTAF2, 20 cultures ΔspTAF2, each done on 1 day accompanied by a total of 10 WT hybrid cultures for normalization	two-sample, two-tailed ttest
S8	spAFG2 swap into Sc, 28C	0.005190866	33 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spAPC1 swap into Sc, 28C	0.854148856	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spCEP3 swap into Sc, 28C	0.501394804	20 cultures swap, done on 1 day accompanied by a total of 10 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spDYN1 swap into Sc, 28C	0.685983102	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spESP1 swap into Sc, 28C	0.003194129	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spMYO1 swap into Sc, 28C	0.000410876	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spSCC2 swap into Sc, 28C	0.010153082	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spTAF2 swap into Sc, 28C	0.420065678	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scAFG2 swap into Sp, 28C	0.212803536	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scAPC1 swap into Sp, 28C	0.008477152	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0

Figure	Comparison	*p*-value	Number of samples	Type of statistical test
	scCEP3 swap into Sp, 28C	0.211757905	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scDYN1 swap into Sp, 28C	7.10E-09	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scESP1 swap into Sp, 28C	0.136933579	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scMYO1 swap into Sp, 28C	3.37E-10	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scSCC2 swap into Sp, 28C	9.59E-07	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scTAF2 swap into Sp, 28C	2.40E-14	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
S10A	spESP1 from Z1 into scD1373, 39C	1.18E-07	4 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spESP1 from IFO1804 into scD1373, 39C	2.04E-16	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spESP1 from N17 into scD1373, 39C	1.52E-17	8 cultures swap, done across 2 days accompanied by a total of 4 cultures of WT Scer for normalization	one-sample, one-tailed ttest for a difference from 1.0
	spESP1 from Z1 into scD1373, 28C	0.003194129	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spESP1 from IFO1804 into scD1373, 28C	0.679833392	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
	spESP1 from N17 into scD1373, 28C	0.823594774	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Scer for normalization	one-sample, two-tailed ttest for a difference from 1.0
S10B	scAPC1 from D1373 into spZ1, 39C	0.027114162	8 cultures swap, done across 4 days accompanied by a total of 8 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scAPC1 from D1788 into spZ1, 39C	0.001447488	12 cultures swap, done across 6 days accompanied by a total of 12 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scAPC1 from YPS128 into spZ1, 39C	0.033387302	10 cultures swap, done across 5 days accompanied by a total of 10 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scAPC1 from D6044 into spZ1, 39C	0.000188618	18 cultures swap, done across 6 days accompanied by a total of 12 cultures of WT Spar for normalization	one-sample, one-tailed ttest for a difference from 1.0
	scAPC1 from D1373 into spZ1, 28C	0.008477152	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scAPC1 from D1788 into spZ1, 28C	2.41E-06	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scAPC1 from YPS128 into spZ1, 28C	0.085740307	22 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0
	scAPC1 from D6044 into spZ1, 28C	0.338841317	33 cultures swap, done on 1 day accompanied by a total of 11 cultures of WT Spar for normalization	one-sample, two-tailed ttest for a difference from 1.0

Supplementary Table 2

Species	Strain name	Background	Genotype/details	Source	Allele donor strain
A. Wild-type diploid and hybrid parent strains					
S. cer	DBVPG1373		soil isolate from Netherlands	SGRP	
S. cer	YPS128		soil isolate from USA	SGRP	
S. cer	DBVPG1788		soil isolate from Finland	SGRP	
S. par	Z1		tree bark isolate from UK	SGRP	
S. par	DBVPG6304		isolate from *Drosophila* from USA	SGRP	
S. par	IFO1804		tree bark isolate from Japan	SGRP	
S. cer	JR501	DBVPG1373	ΔURA3::HygMX/ΔURA3::HygMX	this study	
S. par	JR499	Z1	ΔURA3::HygMX/ΔURA3::HygMX	this study	
S. cer x S. par hybrid	JR507	JR501 x JR499	ΔURA3::HygMX/ΔURA3::HygMX, each S. par and S. cer allele KO'd with HygMX. This is the starting strain for PiggyBac mutagenesis.	this study	
S.cer	DBVPG6044		bili wine isolate from West Africa	SGRP	
S.par	N17		oak exudate isolate from Russia	SGRP	
B. Targeted-deletion reciprocal hemizygote strains					
S. cer x S. par hybrid	CW38	JR507	ΔscAFG2::KanMX/spAFG2	this study	
S. cer x S. par hybrid	CW39	JR507	ΔscAFG2::KanMX/spAFG2	this study	
S. cer x S. par hybrid	CW40	JR507	scAFG2/ΔspAFG2::KanMX	this study	
S. cer x S. par hybrid	CW41	JR507	scAFG2/ΔspAFG2::KanMX	this study	
S. cer x S. par hybrid	CW56	JR507	ΔscCEP3::KanMX/spCEP3	this study	
S. cer x S. par hybrid	CW57	JR507	ΔscCEP3::KanMX/spCEP3	this study	
S. cer x S. par hybrid	CW52	JR507	scCEP3/ΔspCEP3::KanMX	this study	
S. cer x S. par hybrid	CW53	JR507	scCEP3/ΔspCEP3::KanMX	this study	
S. cer x S. par hybrid	CW58	JR507	ΔscMYO1::KanMX/spMYO1	this study	
S. cer x S. par hybrid	CW59	JR507	ΔscMYO1::KanMX/spMYO1	this study	
S. cer x S. par hybrid	CW85	JR507	ΔscMYO1::KanMX/spMYO1	this study	
S. cer x S. par hybrid	CW60	JR507	scMYO1/ΔspMYO1::KanMX	this study	
S. cer x S. par hybrid	CW61	JR507	scMYO1/ΔspMYO1::KanMX	this study	
S. cer x S. par hybrid	CW80	JR507	ΔscSCC2::KanMX/spSCC2	this study	
S. cer x S. par hybrid	CW81	JR507	ΔscSCC2::KanMX/spSCC2	this study	
S. cer x S. par hybrid	CW82	JR507	ΔscSCC2::KanMX/spSCC2	this study	
S. cer x S. par hybrid	CW83	JR507	scSCC2/ΔspSCC2::KanMX	this study	
S. cer x S. par hybrid	CW84	JR507	scSCC2/ΔspSCC2::KanMX	this study	
S. cer x S. par hybrid	CW116	JR507	ΔscESP1::KanMX/spESP1	this study	
S. cer x S. par hybrid	CW122	JR507	ΔscESP1::KanMX/spESP1	this study	
S. cer x S. par hybrid	CW114	JR507	scESP1/ΔspESP1::KanMX	this study	
S. cer x S. par hybrid	CW193	JR507	scESP1/ΔspESP1::KanMX	this study	
S. cer x S. par hybrid	CW131	JR507	ΔscAPC1::KanMX/spAPC1	this study	
S. cer x S. par hybrid	CW160	JR507	ΔscAPC1::KanMX/spAPC1	this study	
S. cer x S. par hybrid	CW185	JR507	scAPC1/ΔspAPC1::KanMX	this study	
S. cer x S. par hybrid	CW189	JR507	scAPC1/ΔspAPC1::KanMX	this study	
S. cer x S. par hybrid	CW172	JR507	ΔscDYN1::KanMX/spDYN1	this study	
S. cer x S. par hybrid	CW173	JR507	ΔscDYN1::KanMX/spDYN1	this study	
S. cer x S. par hybrid	CW112	JR507	scDYN1/ΔspDYN1::KanMX	this study	
S. cer x S. par hybrid	CW113	JR507	scDYN1/ΔspDYN1::KanMX	this study	
S. cer x S. par hybrid	CW213	JR507	ΔscTAF2:NatMX/spTAF2	this study	
S. cer x S. par hybrid	CW214	JR507	ΔscTAF2:NatMX/spTAF2	this study	
S. cer x S. par hybrid	CW221	JR507	scTAF2/ΔspTAF2::NatMX	this study	

Species	Strain name	Background	Genotype/details	Source	Allele donor strain
S. cer x S. par hybrid	CW222	JR507	scTAF2/ΔspTAF2::NatMX	this study	
C. Allele replacement strains					
S. cer	CW64	DBVPG1373	ΔAFG2(-343 to 2390) :: spAFG2(-365 to 2391)	this study	Z1
S. cer	CW65	DBVPG1373	ΔAFG2(-342 to 2391) :: spAFG2(-365 to 2391)	this study	Z1
S. cer	CW66	DBVPG1373	ΔAFG2(-342 to 2391) :: spAFG2(-365 to 2391)	this study	Z1
S. cer	CW73	DBVPG1373	ΔCEP3(-236 to 1831) :: spCEP3(-229 to 1831)	this study	Z1
S. cer	CW74	DBVPG1373	ΔCEP3(-236 to 1840) :: spCEP3(-229 to 1840)	this study	Z1
S. cer	CW104	DBVPG1373	ΔMYO1(-978 to 5848) :: spMYO1(-983 to 5849)	this study	Z1
S. cer	CW105	DBVPG1373	ΔMYO1(-978 to 5848) :: spMYO1(-983 to 5849)	this study	Z1
S. cer	CW98	DBVPG1373	ΔESP1(-384 to 4934) :: spESP1(-361 to 4933)	this study	Z1
S. cer	CW100	DBVPG1373	ΔESP1(-384 to 4934) :: spESP1(-361 to 4933)	this study	Z1
S. cer	CW109	DBVPG1373	ΔSCC2(-405 to 4543) :: spSCC2(-419 to 4543)	this study	Z1
S. cer	CW110	DBVPG1373	ΔSCC2(-377 to 4543) :: spSCC2(-388 to 4543)	this study	Z1
S. cer	CW115	DBVPG1373	ΔAPC1(-525 to 5270) :: spAPC1(-518 to 5270)	this study	Z1
S. cer	CW210	DBVPG1373	ΔAPC1(1626 to 5270) :: spAPC1(1623 to 5270)	this study	Z1
S. cer	CW254	DBVPG1373	ΔDYN1(-284 to 12340) :: spDYN1(-272 to 12340)	this study	Z1
S. cer	CW255	DBVPG1373	ΔDYN1(-284 to 12340) :: spDYN1(-272 to 12340)	this study	Z1
S. cer	CW174	DBVPG1373	ΔTAF2(-831 to 4328) :: spTAF2(-864 to 4319)	this study	Z1
S. cer	CW202	DBVPG1373	ΔTAF2(-831 to 4317) :: spTAF2(-864 to 4308)	this study	Z1
S. par	CW67	Z1	ΔAFG2(-293 to 2391) :: scAFG2(-270 to 2391)	this study	DBVPG1373
S. par	CW69	Z1	ΔAFG2(-293 to 2391) :: scAFG2(-270 to 2391)	this study	DBVPG1373
S. par	CW63	Z1	ΔCEP3(-187 to 1816) :: scCEP3(-194 to 1816)	this study	DBVPG1373
S. par	CW77	Z1	ΔCEP3(-187 to 1816) :: scCEP3(-194 to 1816)	this study	DBVPG1373
S. par	CW124	Z1	ΔMYO1(-907 to 5826) :: scMYO1(-903 to 5824)	this study	DBVPG1373
S. par	CW126	Z1	ΔMYO1(-907 to 5826) :: scMYO1(-903 to 5824)	this study	DBVPG1373
S. par	CW141	Z1	ΔESP1(-418 to 4962) :: scESP1(-439 to 4963)	this study	DBVPG1373
S. par	CW142	Z1	ΔESP1(-418 to 4951) :: scESP1(-439 to 4952)	this study	DBVPG1373
S. par	CW165	Z1	ΔSCC2(-456 to 4556) :: scSCC2(-441 to 4556)	this study	DBVPG1373
S. par	CW166	Z1	ΔSCC2(-456 to 4556) :: scSCC2(-441 to 4556)	this study	DBVPG1373
S. par	CW132	Z1	ΔAPC1(-538 to 5280) :: scAPC1(-545 to 5280)	this study	DBVPG1373
S. par	CW133	Z1	ΔAPC1(-542 to 5275) :: scAPC1(-549 to 5275)	this study	DBVPG1373
S. par	CW169	Z1	ΔTAF2(-806 to 4348) :: scTAF2(-774 to 4357)	this study	DBVPG1373
S. par	CW170	Z1	ΔTAF2(-806 to 4348) :: scTAF2(-774 to 4357)	this study	DBVPG1373
S. par	CW223	Z1	ΔDYN1(-231 to 12377) :: scDYN1(-243 to 12377)	this study	DBVPG1373
S. par	CW224	Z1	ΔDYN1(-231 to 12377) :: scDYN1(-243 to 12377)	this study	DBVPG1373
S. cer	CW284	DBVPG1373	ΔESP1(-384 to 4934) :: spESP1(-361 to 4934)	this study	IFO1804
S. cer	CW288	DBVPG1373	ΔESP1(-384 to 4934) :: spESP1(-361 to 4934)	this study	IFO1804
S. cer	CW287	DBVPG1373	ΔESP1(-384 to 4934) :: spESP1(-361 to 4933)	this study	N17
S. cer	CW324	DBVPG1373	ΔESP1(-384 to 4934) :: spESP1(-361 to 4933)	this study	N17
S.par	CW300	Z1	ΔAPC1(-547 to 5280) :: scAPC1(-554 to 5280)	this study	YPS128
S.par	CW322	Z1	ΔAPC1(-547 to 5280) :: scAPC1(-554 to 5280)	this study	YPS128
S.par	CW307	Z1	ΔAPC1(-547 to 5280) :: scAPC1(-554 to 5280)	this study	DBVPG1788
S.par	CW308	Z1	ΔAPC1(-547 to 5280) :: scAPC1(-554 to 5280)	this study	DBVPG1788
S.par	CW309	Z1	ΔAPC1(-547 to 5280) :: scAPC1(-554 to 5280)	this study	DBVPG6044
S.par	CW310	Z1	ΔAPC1(-547 to 5280) :: scAPC1(-554 to 5280)	this study	DBVPG6044
S.par	CW311	Z1	ΔAPC1(-547 to 5280) :: scAPC1(-554 to 5280)	this study	DBVPG6044

Supplementary Table 3

Sample	Biological Replicate	Technical Replicate	Sample Name	Total Reads
28°C selection	A	1	28A1	76449364
		2	28A2	76081620
		3	28A3	40064389
		4	28A4	32128802
	B	1	28B1	38456506
		2	28B2	42273157
		3	28B3	35808821
		4	28B4	40086768
	C	1	28C1	35599937
		2	28C2	43057928
		3	28C3	40232557
		4	28C4	40422653
39°C selection	A	1	39A1	39511628
		2	39A2	43721972
		3	39A3	39511546
		4	39A4	40650720
	B	1	39B1	42888908
		2	39B2	38950323
		3	39B3	37231294
		4	39B4	38026468
	C	1	39C1	37298412
		2	39C2	37208024
		3	39C3	37982258
		4	39C4	38473876
T0	//	1	T0-1	80596903
		2	T0-2	88916613
		3	T0-3	41141713
		4	T0-4	35890054

Supplementary Table 4

	Forward primer (5’-3’)	Reverse primer (5’-3’)
RH-seq library prep	ATGATACGGCGACCACCGAGATCTACACTCTT TCCCTACACGACGCTCTTCCGATCTNNNNNN AGCAATATTTCAAGAATGCATGCGTCAAT	CAAGCAGAAGACGGCATACGAGATN NNNNNGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT