SUPPORTING INFORMATION

Divergent molecular pathways govern temperature-dependent wheat stem rust resistance genes *Sr6*, *Sr13* and *Sr21*

Tim C. Hewitt[1,2,9], Keshav Sharma[3,9], Jianping Zhang[4,5,9], Prabin Bajgain[6], Dhara Bhatt[1], Jun Yang[1], Smriti Singh[4], Qiaoli Wang[5], Narayana Upadhyaya[1], Curtis Pozniak[7], Robert McIntosh[4*], Evans Lagudah[1*], Peng Zhang[4*] & Matthew Rouse[3,8*]

[1]CSIRO Agriculture & Food, GPO Box 1700, Canberra, ACT 2601, Australia.

[2]Present address: Immunology & Infectious Diseases, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia.

[3]USDA-ARS, Cereal Disease Laboratory, 1551 Lindig Street, St. Paul, MN 55108, USA.

[4]University of Sydney, Plant Breeding Institute, School of Life and Environmental Sciences, 107 Cobbitty Road, Cobbitty, NSW 2570, Australia.

[5]State Key Laboratory of Wheat and Maize Crop Science, National Wheat Innovation Centre, Centre for Crop Genome Engineering, and College of Agronomy, Longzi Lake Campus, Henan Agricultural University, Zhengzhou 450046, China.

[6]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA.

[7]Crop Development Centre and Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK S7N 5A8, Canada.

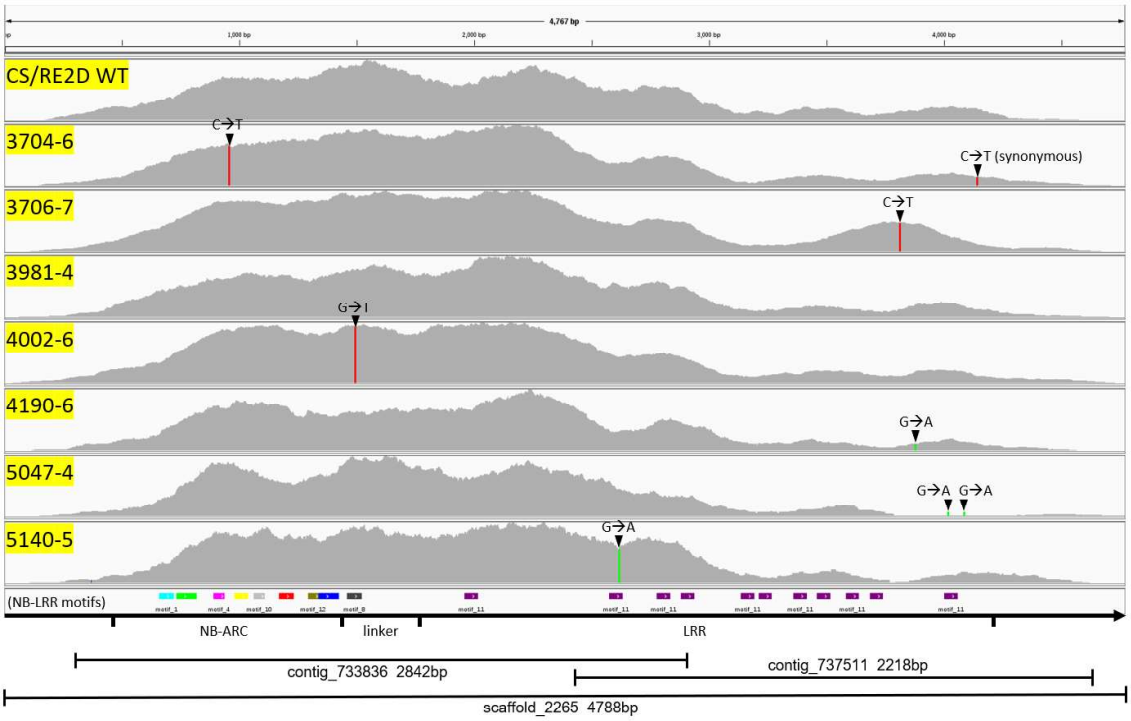
[8]Present address: USDA-ARS, Sugarcane Field Station, 12990 U.S. Highway 441 N, Canal Point, FL 33438, USA.

[9]These authors contributed equally: Tim Hewitt, Keshav Sharma, Jianping Zhang

[*]Corresponding authors: robert.mcintosh@sydney.edu.au; evans.lagudah@csiro.au;

peng.zhang@sydney.edu.au; Matthew.rouse@usda.gov

A)



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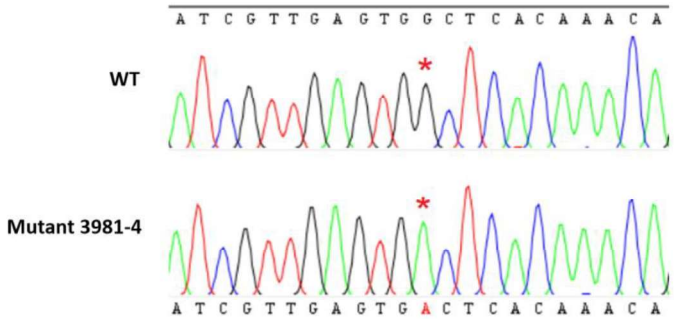
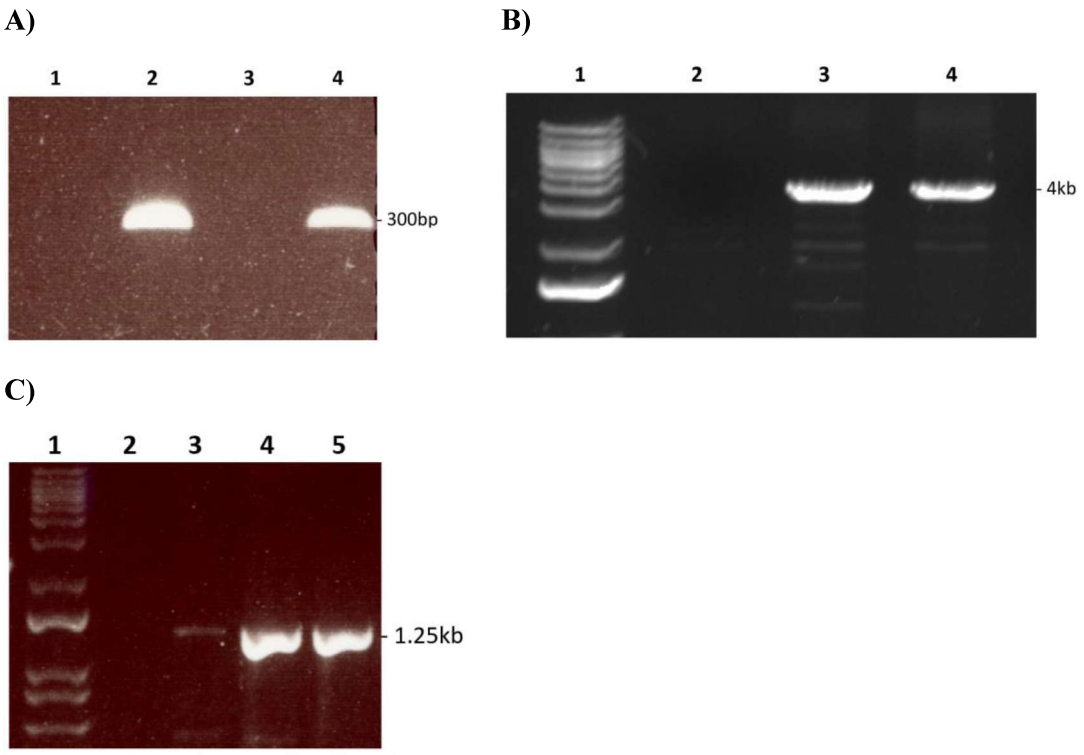
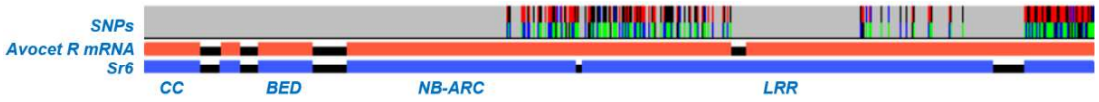


Supplementary Fig. 1. Identification of resistance knockout mutations from mutant sequencing data. A: Histograms of RenSeq read alignments to candidate contigs showing mutation positions. Coverage depth of read alignments to candidate sequences is indicated in grey. Sample IDs are highlighted in yellow. SNPs are denoted with red or green vertical bars that indicate a mismatch with the wildtype (WT) occurring in almost 100% of the overlapping reads. Specific base changes are labelled. Placements of independently assembled contigs are shown. **B:** Region covering a mutation that was not enriched by RenSeq. Position of SNP is indicated by asterisks.

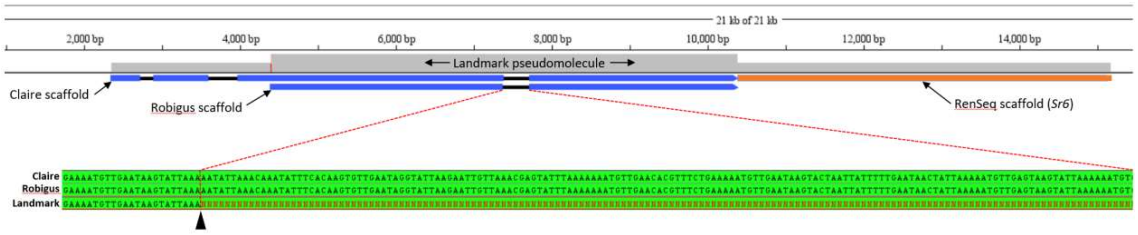


Supplementary Fig. 2. PCR screens from different stages of *Sr6* cloning work. A: Dominant STS marker diagnostic for *Sr6*. *Sr6STS1* was validated in 197 F_3 lines from reciprocal crosses of Chinese Spring and CS/Red Egyptian (RE) 2D substitution line. Agarose gel lanes: 1=CS, 2=CS/RE 2D, 3=Avocet R, 4=Manitou. **B:** PCR product of the main coding region covered by the longest scaffold. Agarose gel lanes: 1=ladder, 2=CS, 3=CS/RE 2D, 4=mutant 5047-4. **C:** PCR product of upstream coding region carrying CC and BED domains. Agarose gel lanes: 1=ladder, 2=water, 3=CS, 4=CS/RE 2D, 5=mutant 3981-4.



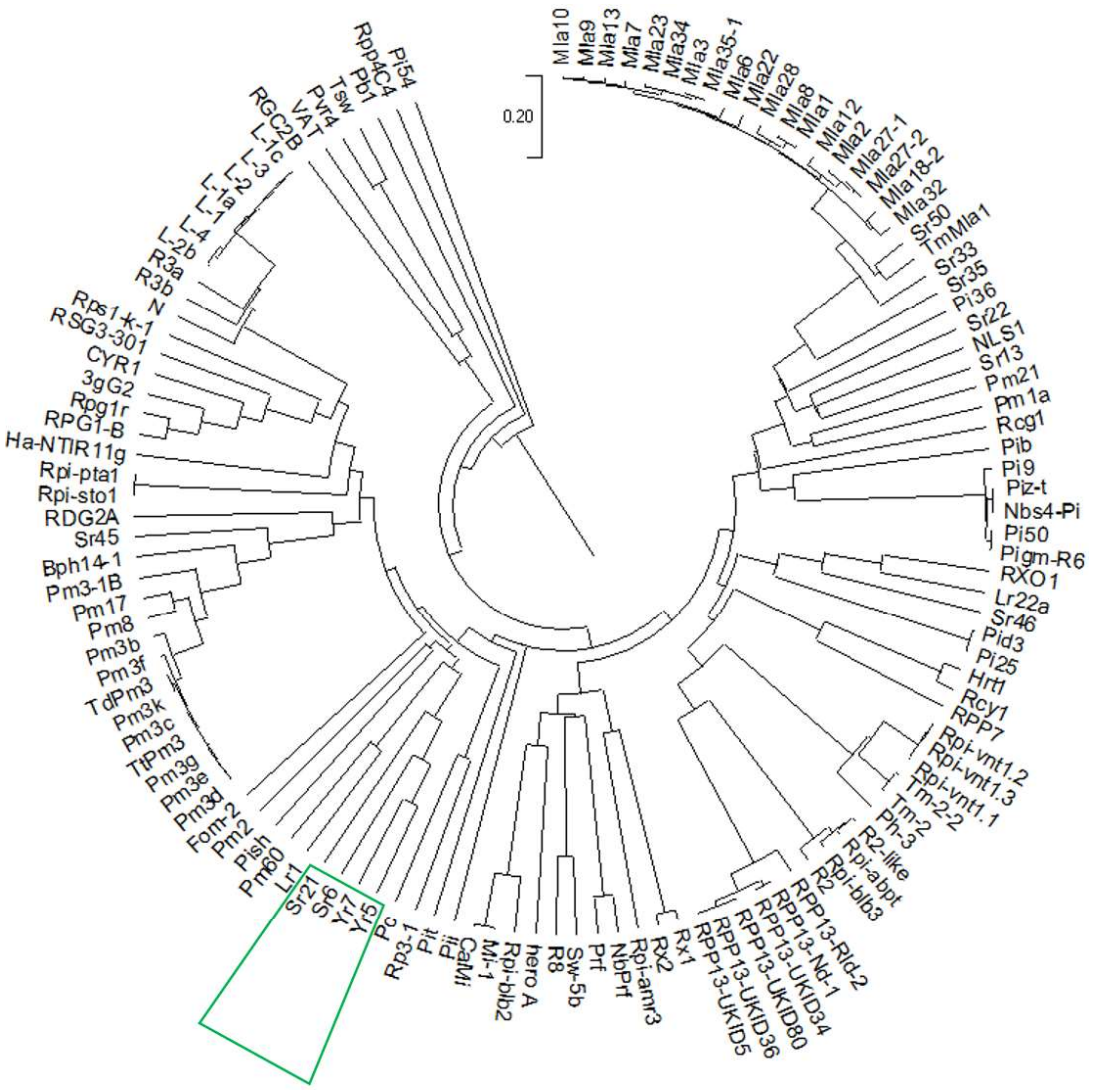
Supplementary Fig. 3. Alignment of *Sr6* and Avocet R transcripts against Landmark reference genome. Transcript of *Sr6* showing structure conservation with the transcript from Avocet R RNAseq. Mismatches with Avocet R are indicated by coloured bars. Gaps in the LRR region of *Sr6* denote extra sequence insertions in Avocet R.

A)

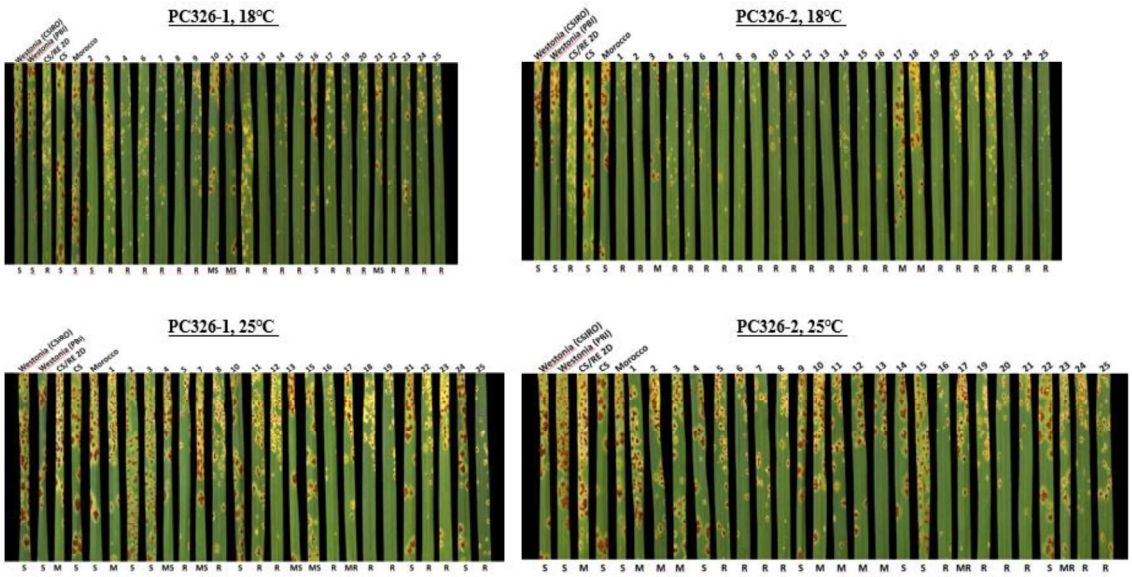


B)

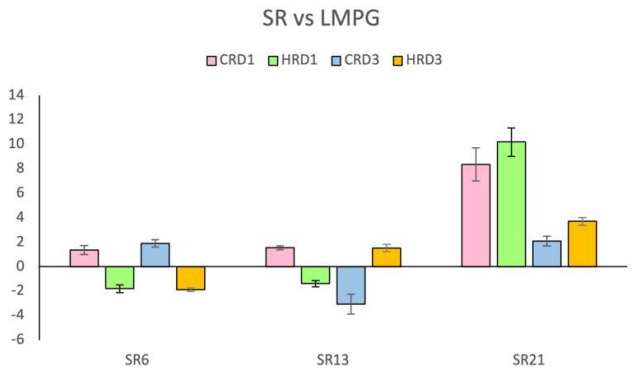
Supplementary Fig. 4. Comparison of *Sr6* sequences to other accessions. A: Gap closing upstream region of *Sr6* by aligning scaffolds from different assemblies. Scaffolds aligned to Landmark reference genome. The start of the gap interval in Landmark is expanded and indicated by black arrowhead. **B:** Amino acid sequence alignments of BED domains from *Sr6*, *Yr5* and *Yr7*. Conserved residues with *Sr6* are highlighted in colour.



Supplementary Fig. 5. Phylogenetic tree showing *Sr6* among various plant CNL type NB-LRRs. The optimal tree with the sum of branch length = 30.56 is shown. The tree is drawn to scale and the analysis involved 125 amino acid sequences. The group containing *Sr6* is boxed in green.

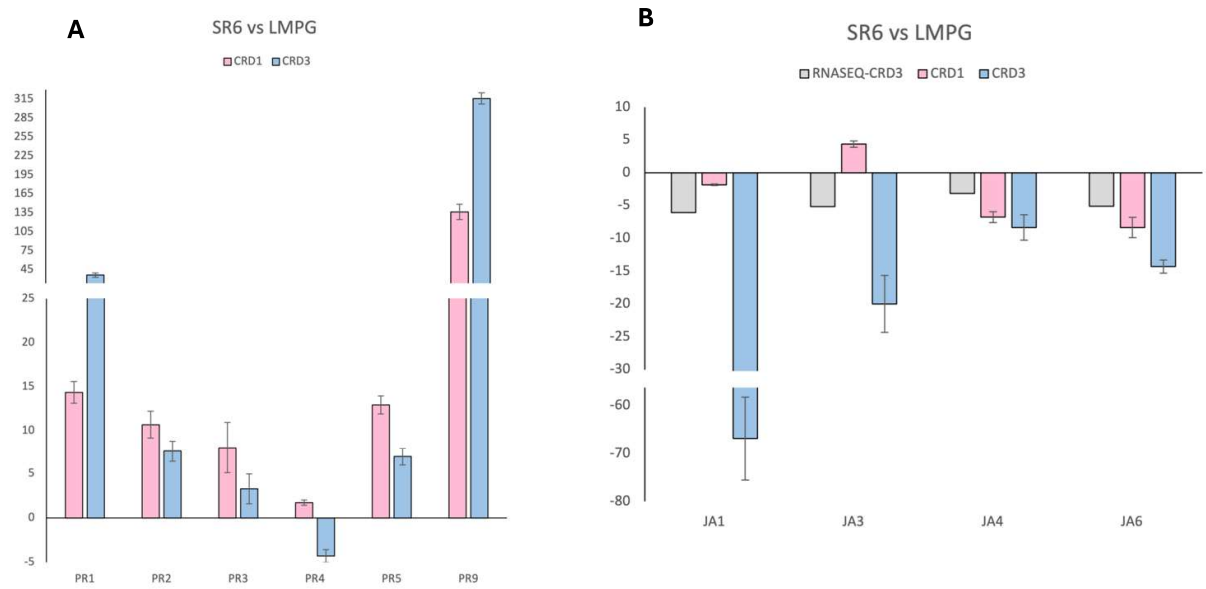


Supplementary Fig. 6. Westonia+*Sr6* transgenic T1 seedling leaves at 14 days post-inoculation with *Pgt* 21-0. All numbered individuals were derived from two T_0 parents (PC326-1, PC326-2) and grown at either 18°C (upper panel) or 25°C (lower panel). *Sr6*+ control: CS/RE 2D; *Sr6*- controls: Westonia (CSIRO and PBI accessions), CS, Morocco. Bottom labels indicate disease phenotype scored as resistant (R), moderately resistant (MR), moderately resistant/moderately susceptible (M), moderately susceptible (MS), or susceptible (S).



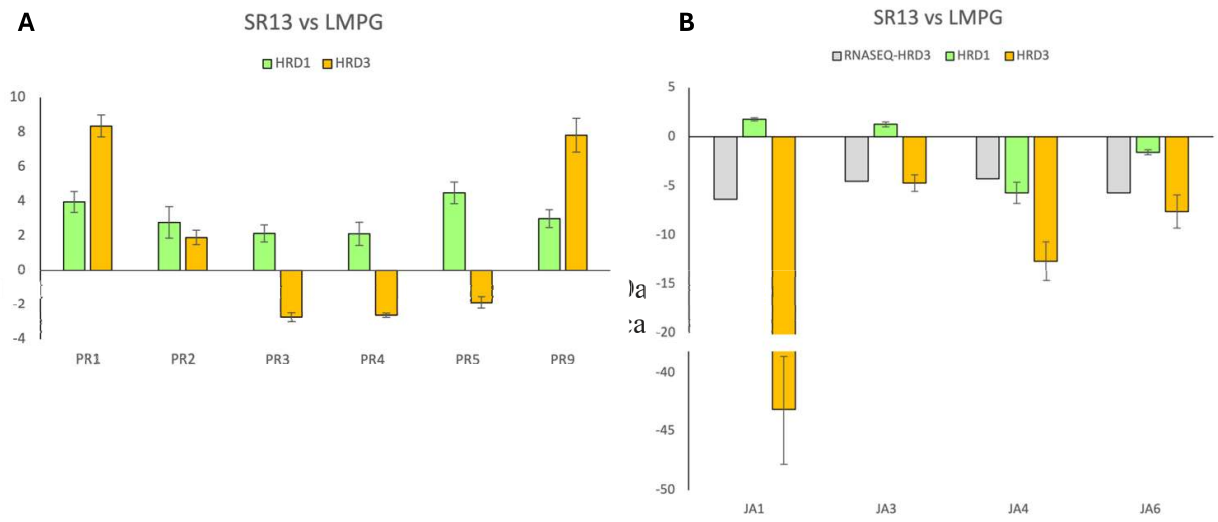
Note: High temp Day 1=light green, High temp Day 3=orange. Low temp Day 1= rose, Low temp Day 3 = light blue. The relative transcript abundance considered statistically significant with log fold change ± 1.5 and P value of < 0.05.

Supplementary Fig. 7.1. Relative transcript abundance of *Sr6*, *Sr13* and *Sr21* genes in their respective lines. *Sr6* is upregulated at low and downregulated at high temperature. *Sr13* is upregulated under low temperature conditions at Day 1 and under high temperature conditions at day 3. *Sr21* is upregulated at all tested temperature conditions and time points.

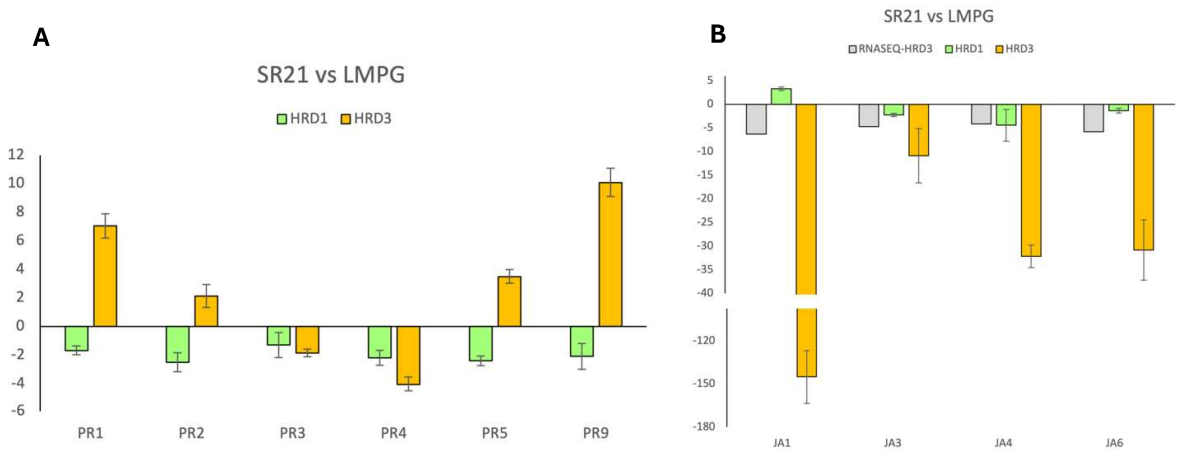


Supplementary Fig. 7.2. qPCR validation of pathogenesis related (PR) genes (PR1, PR2, PR3, PR4, PR5 and PR9) and JA genes (JA1, JA3, JA4 and JA6) that are expressed in *Pgt* infected SR6 lines at low temperature. The expression of PR (A) and JA (B) genes was validated with qPCR. The expression of PR genes follows similar expression pattern at Day1 and Day3 and almost all the tested candidates are upregulated. From the four tested JA genes, JA3 is upregulated at Day1 but all other genes are downregulated. At Day3 all four JA genes are downregulated (RNAseq) and validated with qPCR. The JA genes mostly follow the same expression pattern and are downregulated.

Note: Low temp day 1= Rose, low temp day3=Light blue, CR=low temp rust infected (18-15°C). Relative transcript abundance is considered statistically significant with log fold change ± 1.5 and P value of < 0.05.

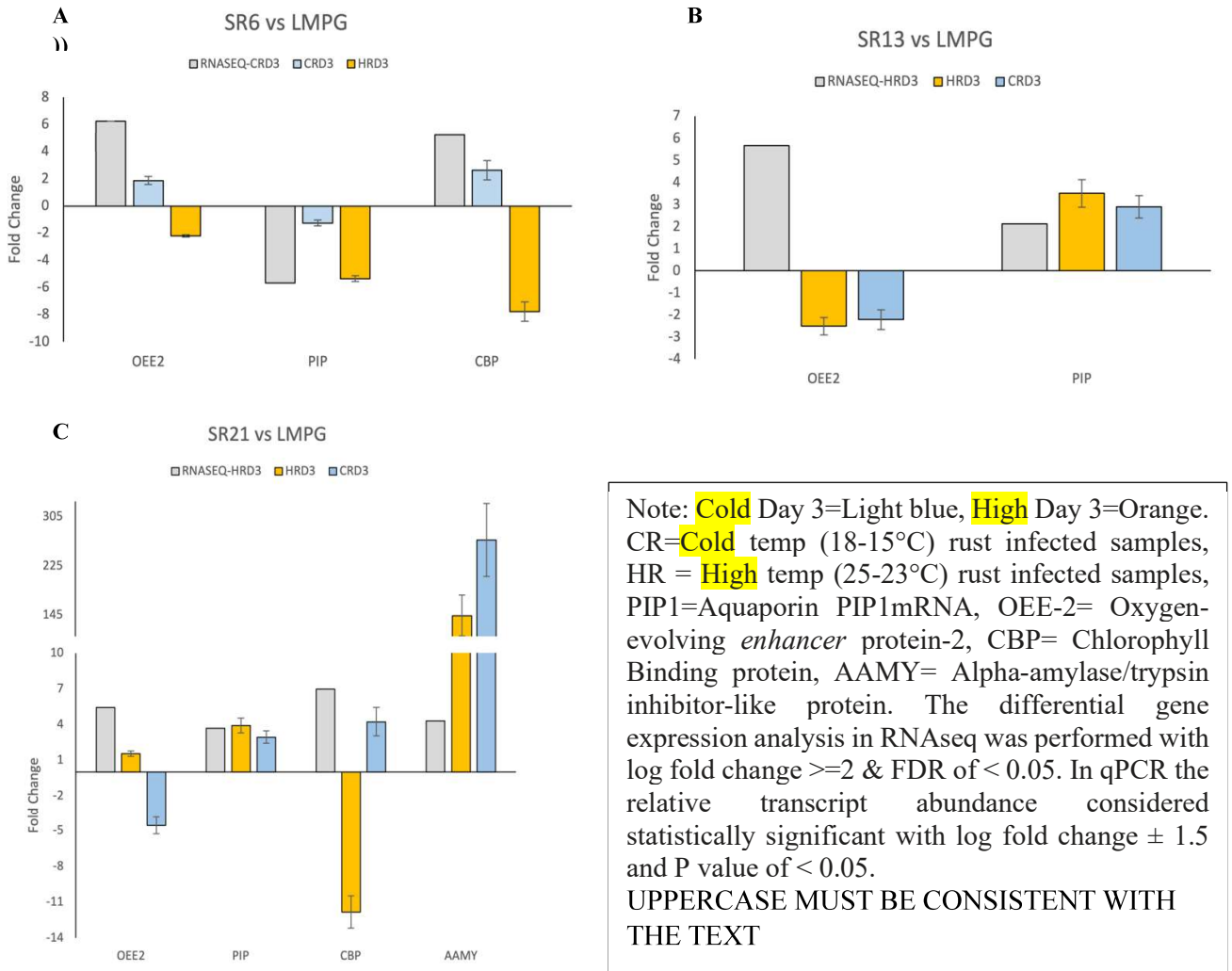


Supplementary Fig. 7.3. Relative expression of the PR and JA genes expressed in *Pgt*-infected lines with *Sr13*. Gene expression was validated with qPCR for Day 1 (light green) and Day 3 (orange) relative to transcript abundance in LMPG. **A:** Among the six tested PR genes (*PR1*, *PR2*, *PR3*, *PR4*, *PR5*, *PR9*), all are upregulated at Day 1 whereas *PR3*, *PR4* and *PR5* are downregulated at Day 3. **B:** Two JA genes, *JA1* and *JA3*, are upregulated at Day 1. All JA genes are downregulated at Day 3 [RNAseq (grey) and qPCR (orange)].



Supplementary Fig. 7.4. Fold expression of PR (*PR1*, *PR2*, *PR3*, *PR4*, *PR5* and *PR9*) and JA (*JA1*, *JA3*, *JA4* and *JA6*) genes expressed in *Pgt* infected lines with *Sr21* at Day 1 and Day 3. The expression of PR (A) and JA (B) genes were validated with qPCR. All PR genes except *PR3* and *PR4* are upregulated at Day 1, but all are downregulated at Day 3. JA genes are downregulated at both Day 1 and Day3 except *JA1* which is upregulated at Day 1. Relative transcript abundance is considered statistically significant with log fold change ± 1.5 and P value of < 0.05.

Note: Day 1=light green, Day3=orange, HR=High temp rust-inoculated (25-23°C). Relative transcript abundance is considered statistically significant with log fold change ± 1.5 and P value of < 0.05.



Note: Cold Day 3=Light blue, High Day 3=Orange. CR=Cold temp (18-15°C) rust infected samples, HR = High temp (25-23°C) rust infected samples, PIP1=Aquaporin PIP1mRNA, OEE-2= Oxygen-evolving *enhancer* protein-2, CBP= Chlorophyll Binding protein, AAMY= Alpha-amylase/trypsin inhibitor-like protein. The differential gene expression analysis in RNAseq was performed with log fold change >=2 & FDR of < 0.05. In qPCR the relative transcript abundance considered statistically significant with log fold change ± 1.5 and P value of < 0.05.
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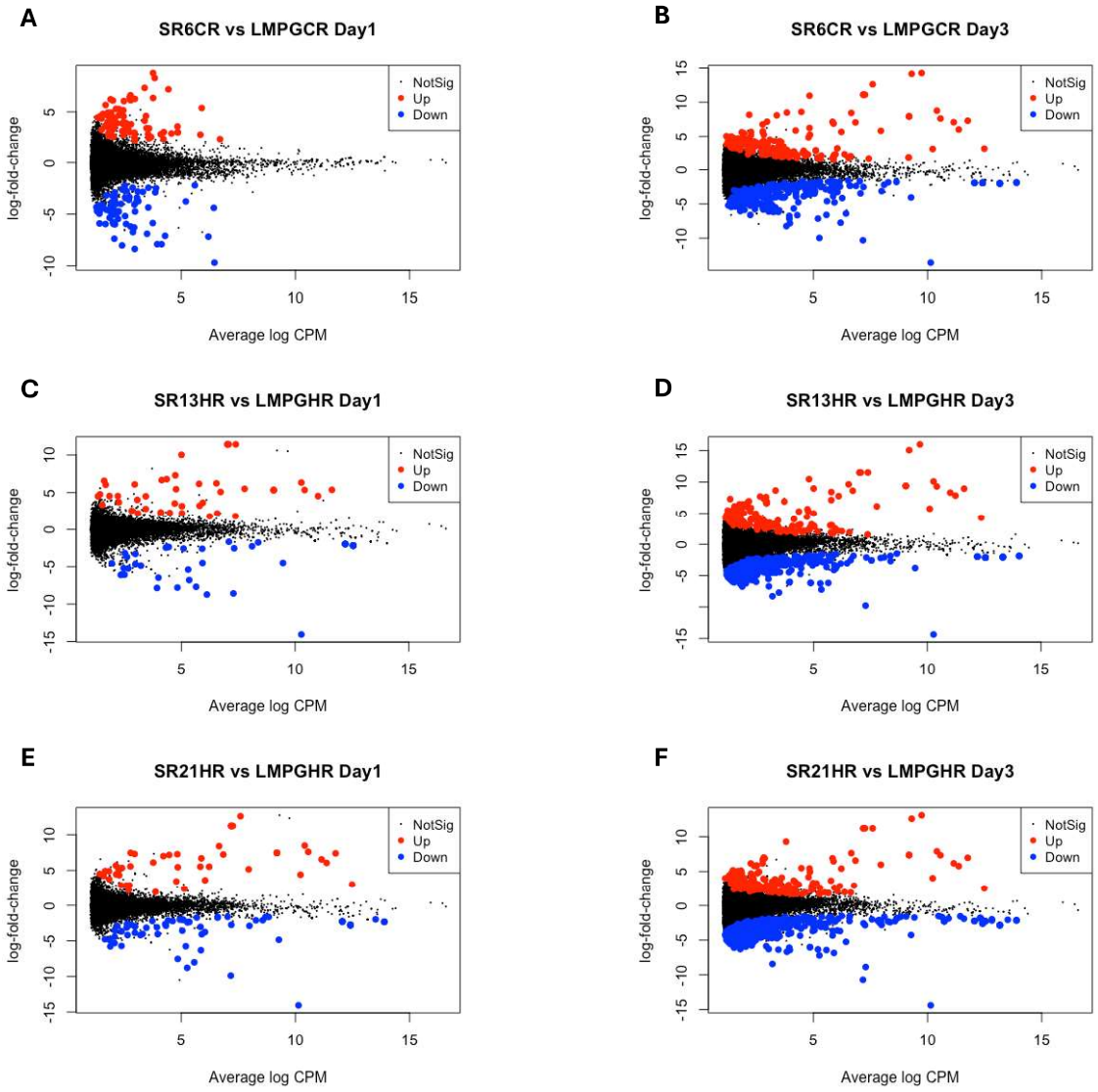
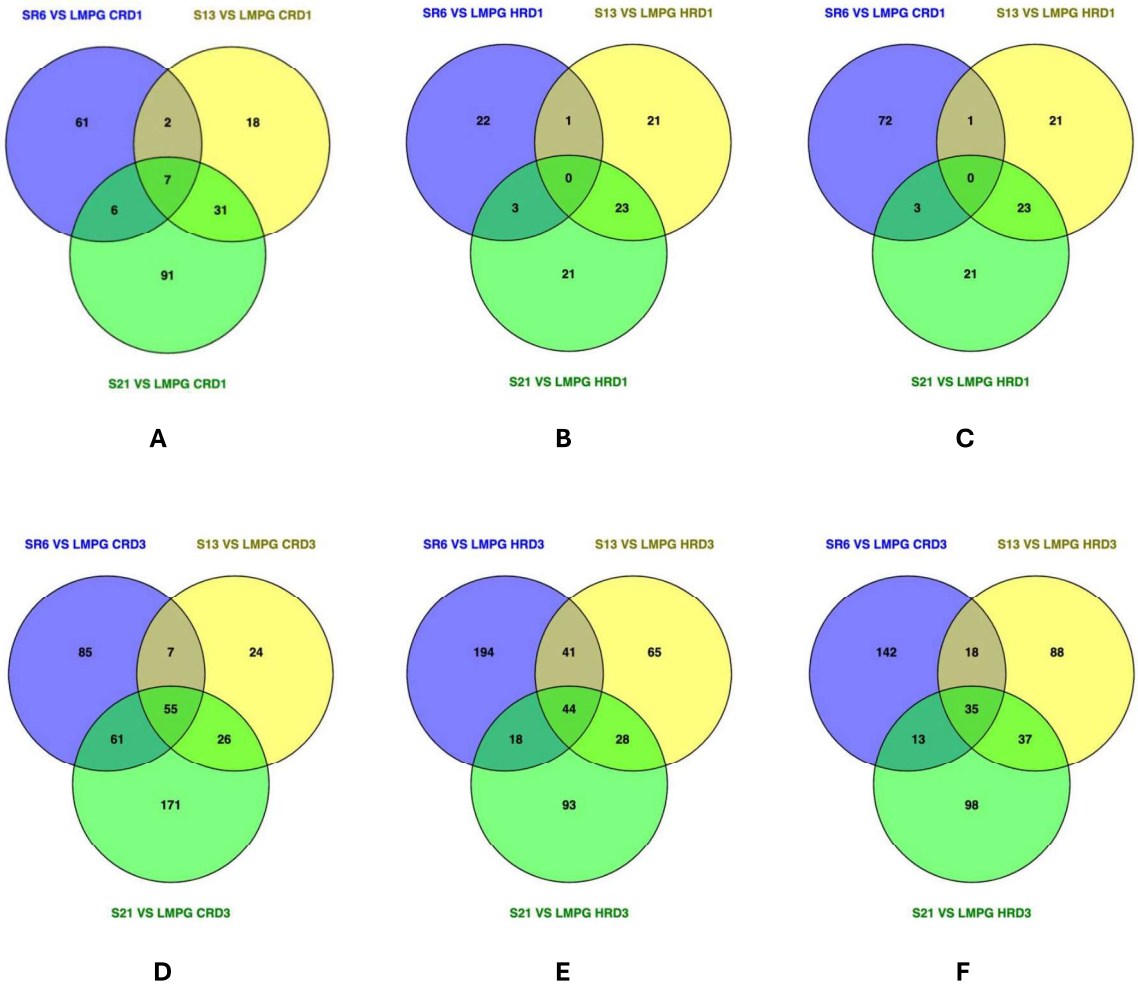
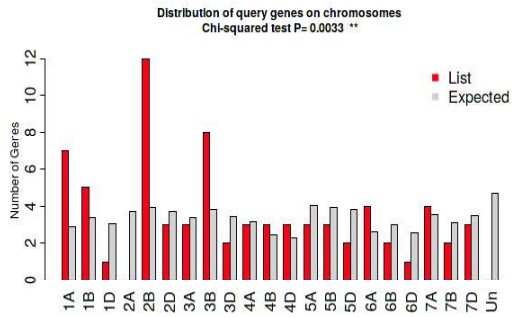
Supplementary Fig. 7.5. Confirmation of the relative transcript abundance in *Pgt* infected *Sr6*, *Sr13* and *SR21* lines at day3 through RNAseq and qPCR analyses. Gene validation performed through qPCR (colored) and RNAseq (grey). **A:** SR6 vs LMPG Day3. OEE-2 and CBP are upregulated at low temperature but downregulated at high temperature. PIP1 is downregulated at both high and low temperatures. qPCR values are compared to RNAseq data (fold expression) at low temperature. OEE-2 and CBP are upregulated, and PIP1 is downregulated in RNAseq analysis. **B:** SR13 vs LMPG Day3. In RNAseq, PIP1 and OEE-2 are upregulated at both low and high temperatures. qPCR analysis shows PIP1 is upregulated and OEE-2 is downregulated at both temperatures. **C:** SR21 vs LMPG Day3. RNAseq analysis shows all four selected genes are upregulated. qPCR analysis shows PIP1 and AAMY are upregulated at both low and high temperatures, and OEE-2 and CBP are downregulated at low and high temperature, respectively. AAMY is significantly upregulated at all tested temperature conditions.



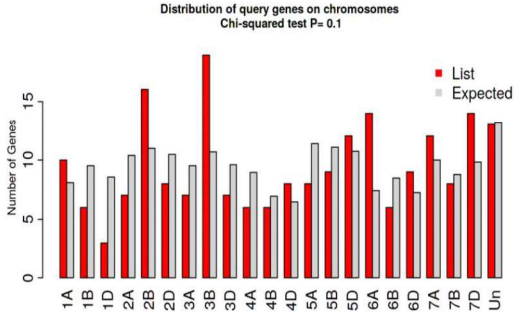
Supplementary Fig. 8. Volcano plot distribution of differential gene expression in LMPG near-isogenic lines compared to LMPG. A: LMPG-*Sr6* at low temperature at day 1. **B:** LMPG-*Sr6* at low temperature at day 3. **C:** LMPG-*Sr13* at high temperature at day 1. **D:** LMPG-*Sr13* at high temperature at day 3. **E:** LMPG-*Sr21* at high temperature at day 1. **F:** LMPG-*Sr21* at high temperature at day 3. The significantly differentially expressed genes (log fold change >= 2 and FDR (false discovery rate) of <0.05) are represented by red (upregulated) or blue (downregulated) dots. Black dots represent non-significant genes. X axis represents log fold change and Y axis represents average counts per million.



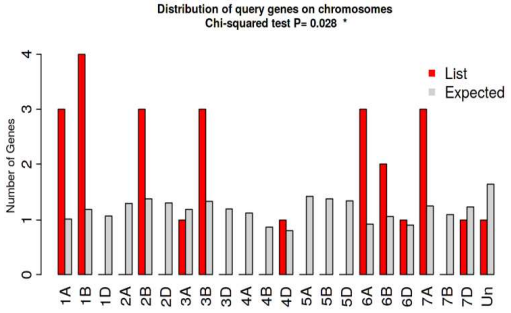
Supplementary Fig. 9. Venn Diagrams indicating the number of upregulated genes in LMPG-*Sr6*, LMPG-*Sr13*, and LMPG-*Sr21* relative to LMPG at **A:** low temperature at day 1, **B:** high temperature at day 1, **C:** temperatures where each temperature-sensitive gene is most effective at day 1, **D:** low temperature at day 3, **E:** high temperature at day 3, and **F:** temperatures at which each temperature-sensitive gene is most effective at day 3.



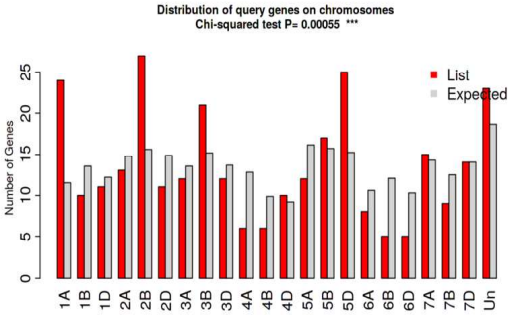
A: SR6CRD1 VS LMPGCRD1



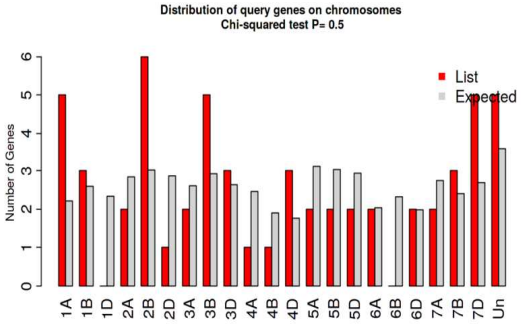
B: SR6CRD3 VS LMPGCRD3



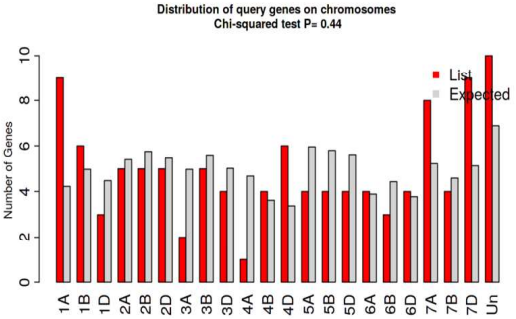
C: SR6HRD1 VS LMPGHRD1



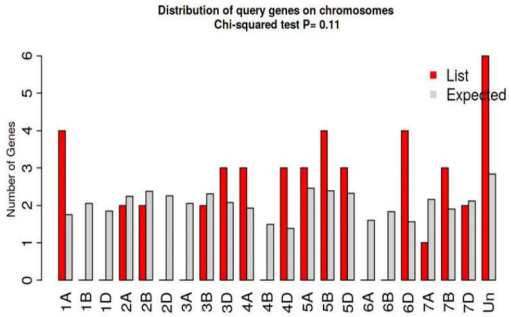
D: SR6HRD3 VS LMPGHRD3



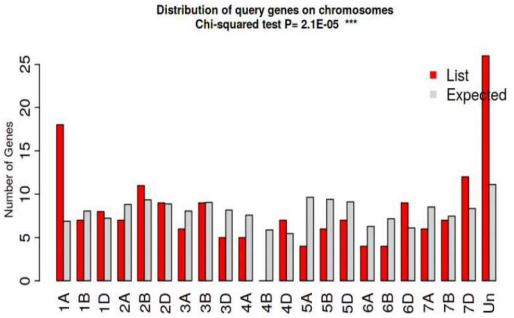
E: SR13CRD1 VS LMPGCRD1



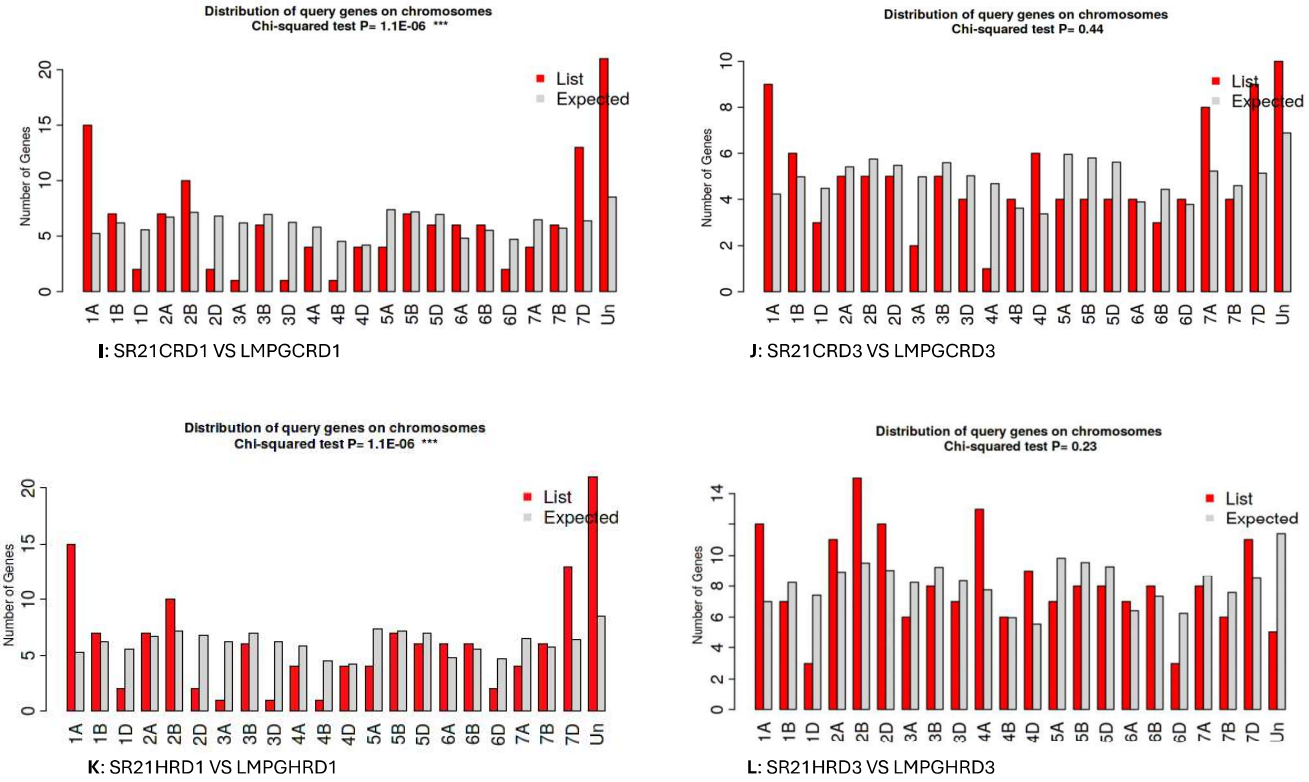
F: SR13CRD3 VS LMPGCRD3



G: SR13HRD1 VS LMPGHRD1

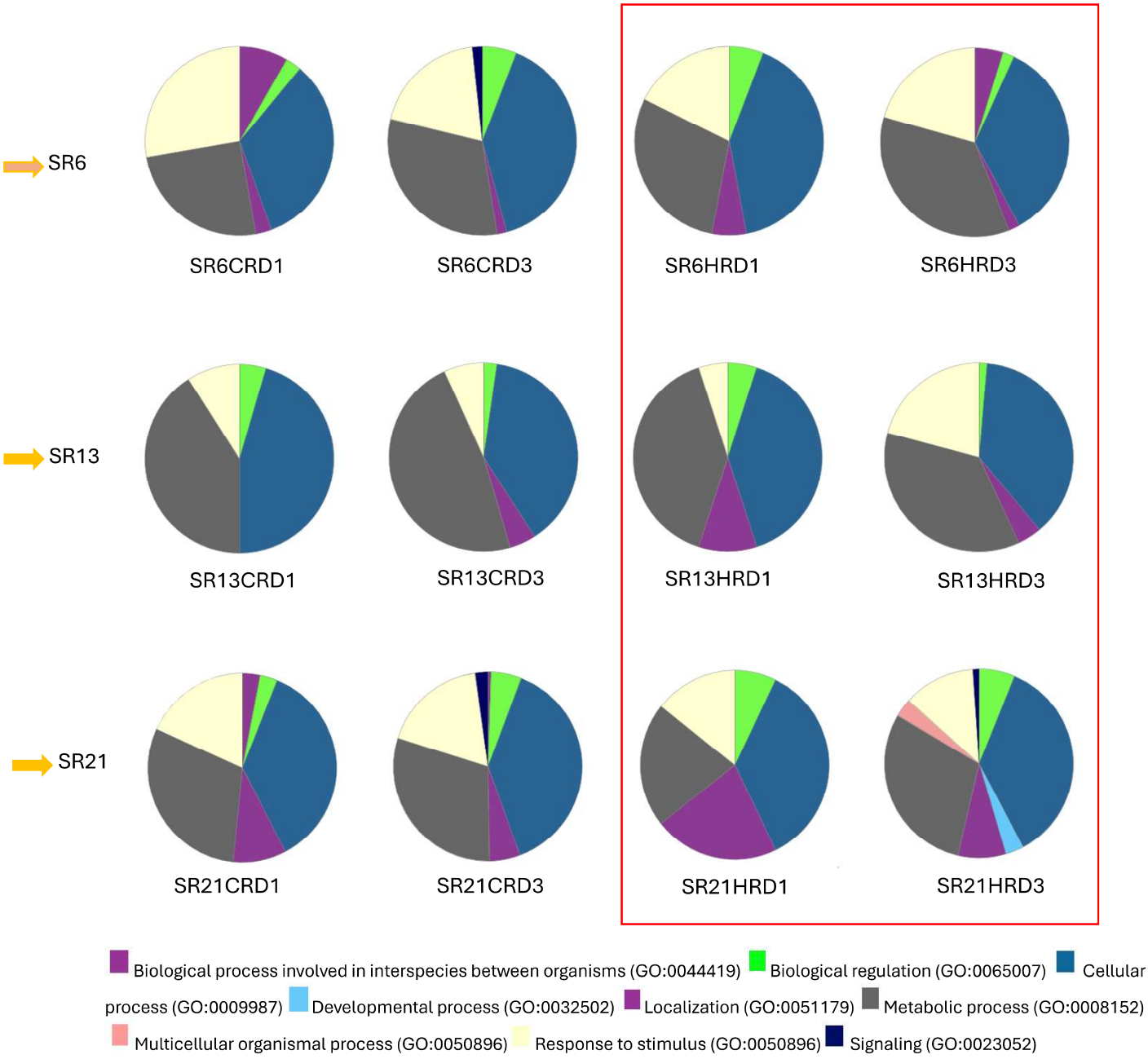


H: SR13HRD3 VS LMPGHRD3



Note: CR= Low temp, rust inoculated; HR= High temp, rust Inoculated

Supplementary Fig. 10. Distribution of genes upregulated in *Sr*-containing lines and downregulated in LMPG across wheat chromosomes. Red color refers to the DEGs mapping to the chromosome and grey color is the expected distribution of the genes across chromosomes. Y axis indicates the number of genes and x axis shows chromosome number including unmapped reads at the end.



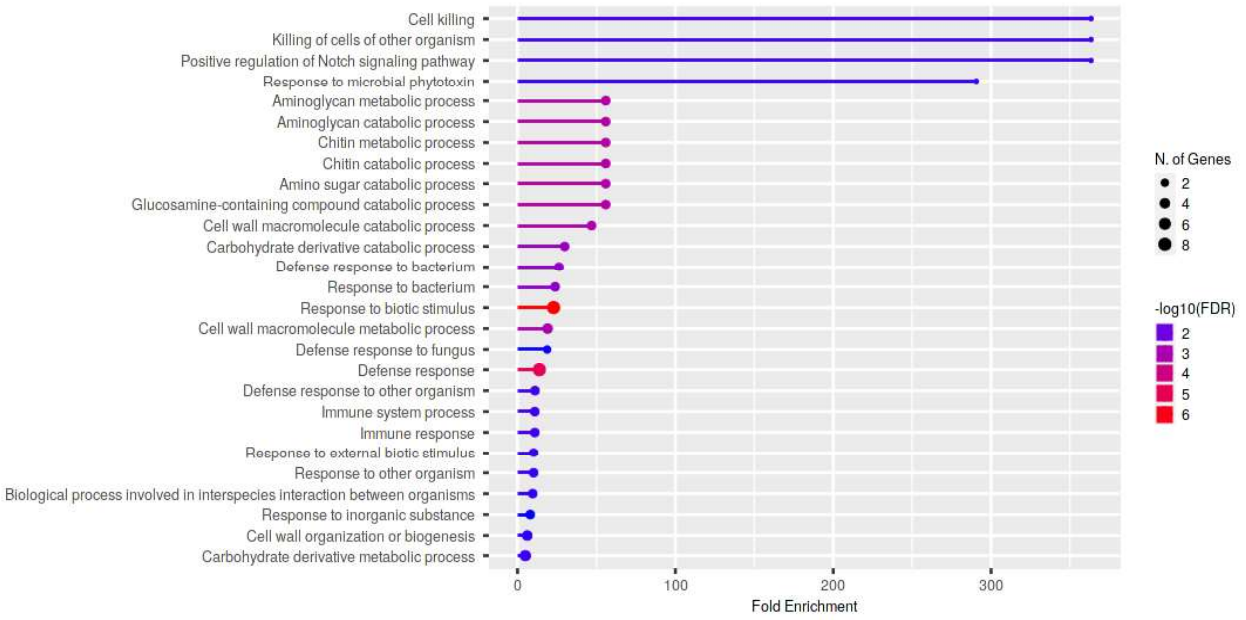
Note: CR=Low temp, rust inoculated; HR=High temp, rust inoculated, D1=Day 1, D3=Day 3. Each color represents genes associated with cellular functions or pathways. Charts inside the red box correspond to the high temperature condition and outside the red box correspond to the low temperature condition.

Supplementary Fig. 11. Pie chart distribution of differentially expressed genes in lines with *Sr* genes.
Sr6: About one fourth of genes in the *Sr6* line are associated with response to stimulus (yellow). Genes associated with signaling (dark blue) are only seen under low temperature at Day 3. Number of genes associated with biological processes (light green) is higher at Day 3 under low temperature but is low under high temperature conditions. Genes with interspecies interaction (purple) are seen at Day 1 at low temperature and at Day 3 under high temperature. Genes associated to metabolic processes are higher at Day 3 under both temperature conditions. Significant number of genes involving in cellular processes (blue) are differentially expressed

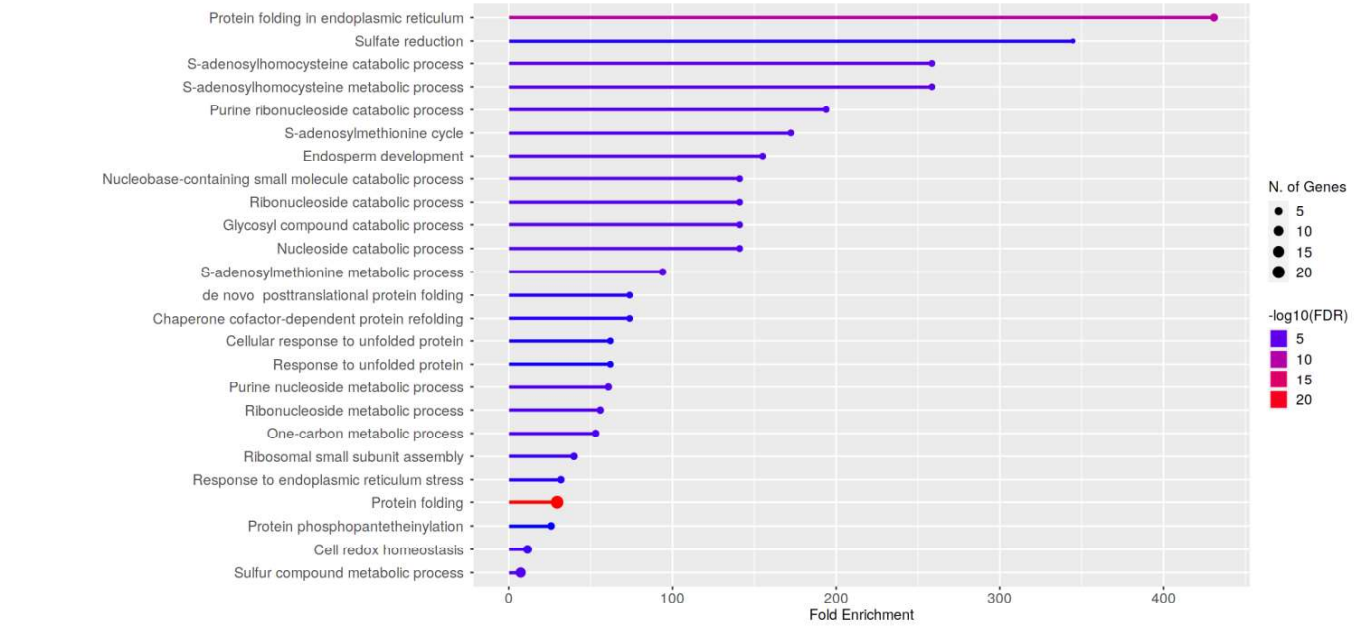
in all conditions. The localization genes (purple 2nd) are higher in Day 1 and are gradually reduced at Day 3 in all conditions.

Sr13: Response to stimulus genes (yellow) are higher at Day 1 but slightly reduced at Day 3 under low temperature conditions. Under high temperature these genes are drastically increased at Day 3. Biological regulation (light green) genes are reduced at Day 3 in both temperature conditions. Cellular (blue) and metabolic (gray) process genes are higher under low temperature conditions. Localization (purple 2nd) genes missing under low temperature are expressed under high temperature at Day 1; a similar number of genes are expressed at Day 3 at both conditions.

Sr21: Response to stimulus genes (yellow) are slightly higher under low temperature conditions. Genes associated with signaling (dark blue) are observed only at Day 3 and the number is higher under low temperature. Genes associated with biological processes involved in interspecies between organisms (purple) are only seen under low temperature and the number is higher at Day 1. Biological regulation genes (light green) are observed under all conditions and the number is slightly higher under high temperature. The number of localization (purple 2nd) genes are higher at Day 1 and lower at Day 3 under both temperature conditions but higher in the high temperature compared to low temperature conditions. One-third of the cellular process genes (blue) are expressed under all conditions. The number of metabolic process (grey) genes are almost similar under low temperature but higher at Day 3 under high temperature. Developmental process (light blue) and multicellular organismal process (pink) genes are only observed at Day 3 under high temperature conditions.

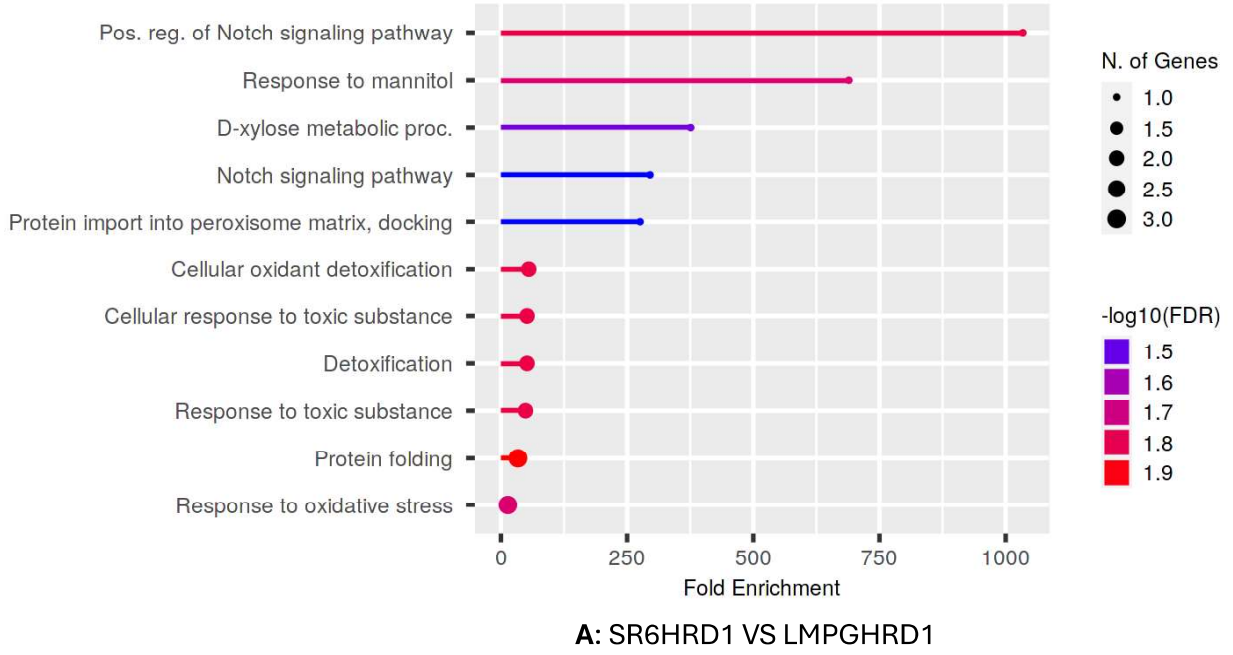


A: SR6CRD1 VS LMPGCRD1

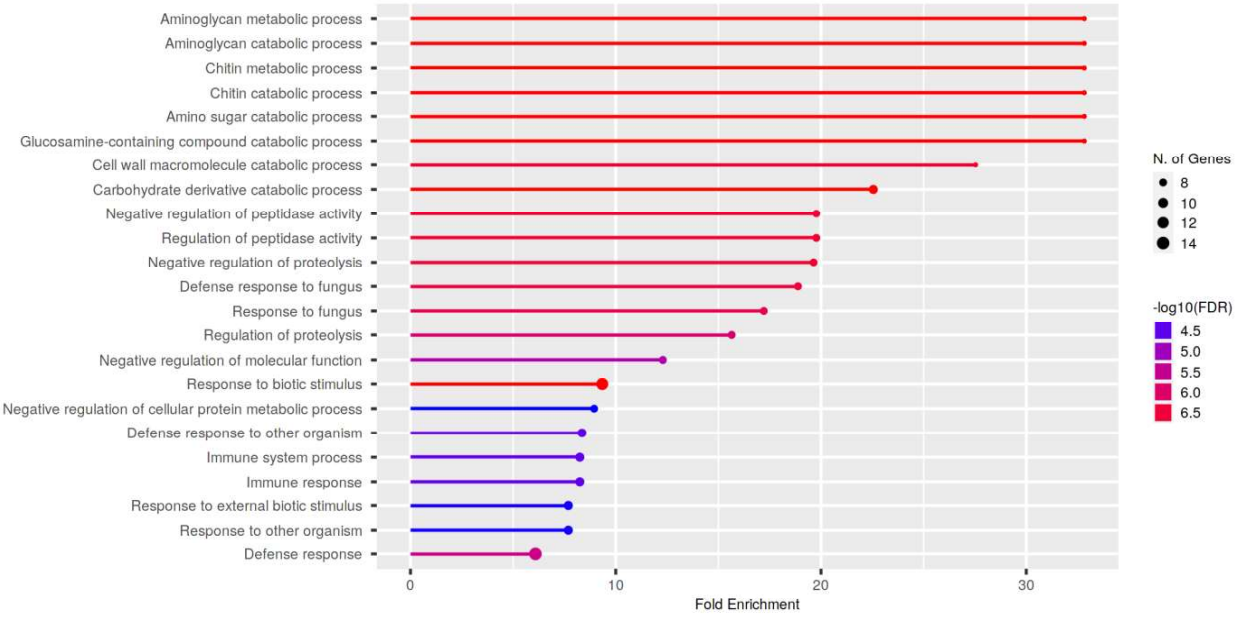


B: SR6CRD3 VS LMPGCRD3

Supplementary Fig. 12. Fold enrichment analysis of genes upregulated in LMPG+*Sr6* under low temperature. A: Genes/pathways expressed at Day 1**, B:** Genes/pathways expressed at Day 3. Round dots represent the number of genes, where larger size represents a higher number of genes. X axis represents the fold enrichment calculated based on the number of genes associated with the pathways/biological processes. Y axis represents pathway. Red color represents the most significant process and blue are less significant in terms of -log10(FDR).

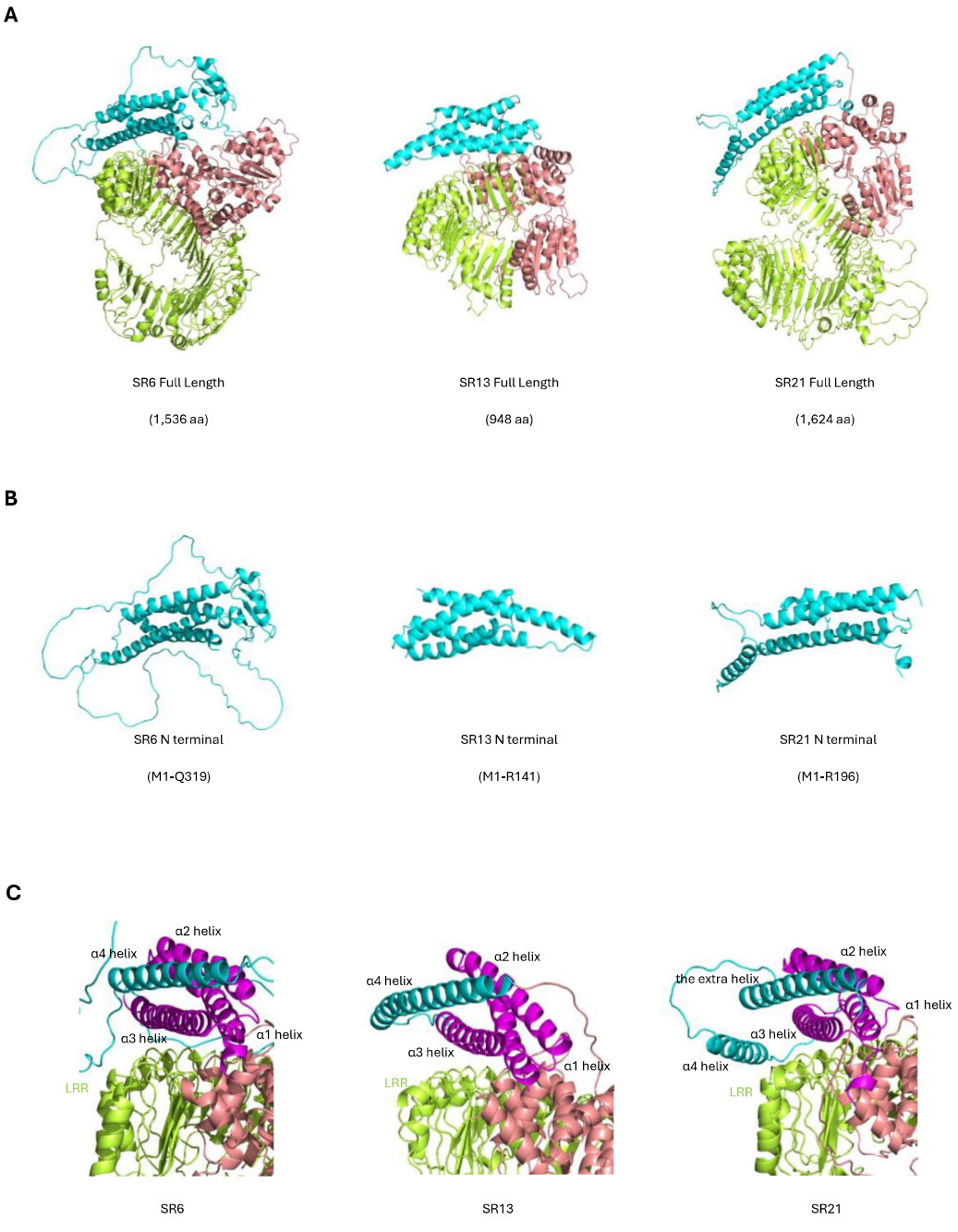


A: SR6HRD1 VS LMPGHRD1

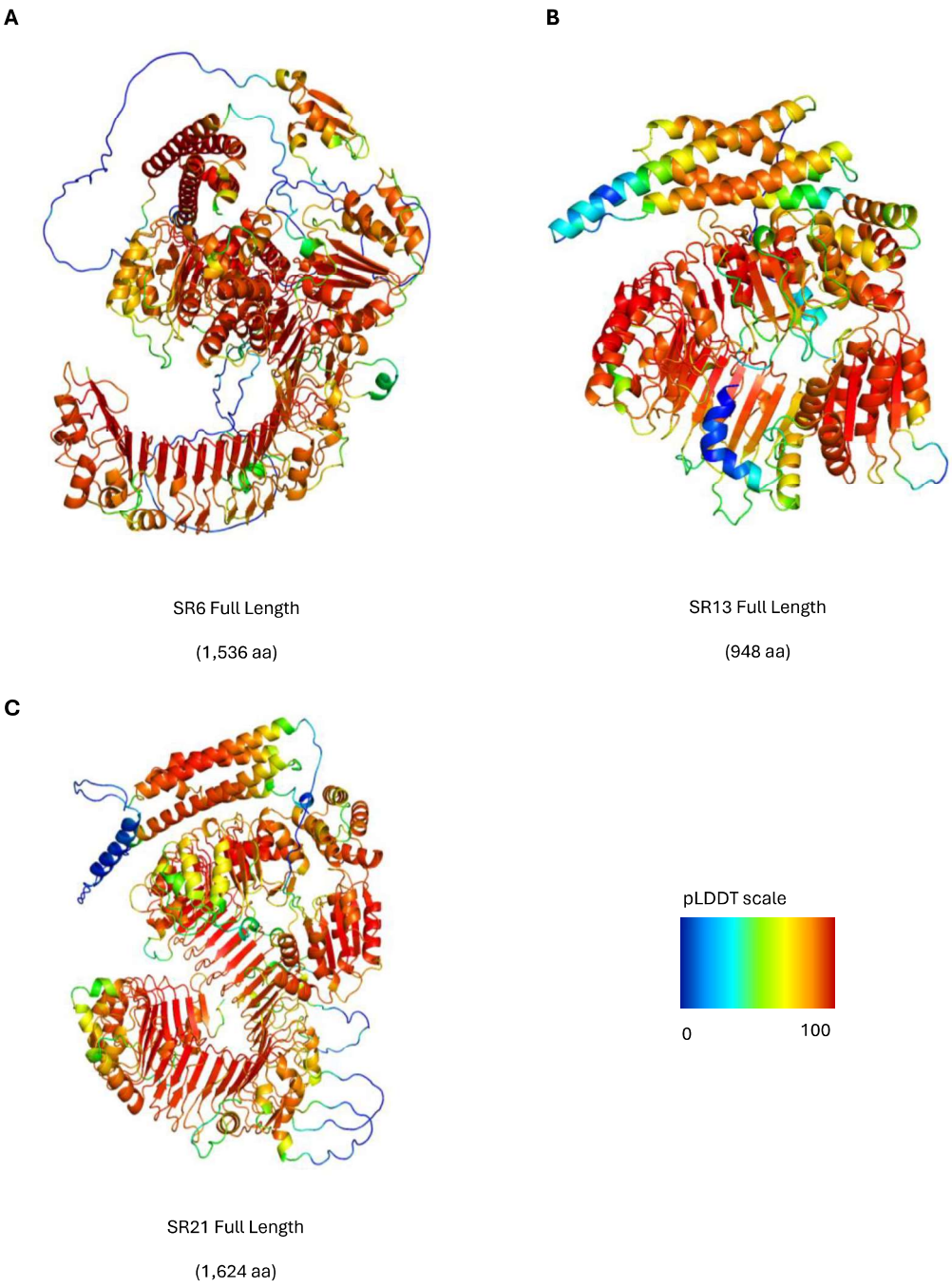


B: SR6HRD3 VS LMPGHRD3

Supplementary Fig. 13. Fold enrichment analysis of genes upregulated in LMPG+*Sr6* under high temperature. A: Genes/pathways expressed at Day 1, **B:** Genes/pathways expressed at Day 3. Round dots represent the number of genes where larger dots represent a higher number of genes. X axis represents the fold enrichment calculated based on the number of genes associated with the pathways/biological process. Y axis represents pathway. Red color represents the most significant process and blue are the less significant in terms of -log10(FDR).

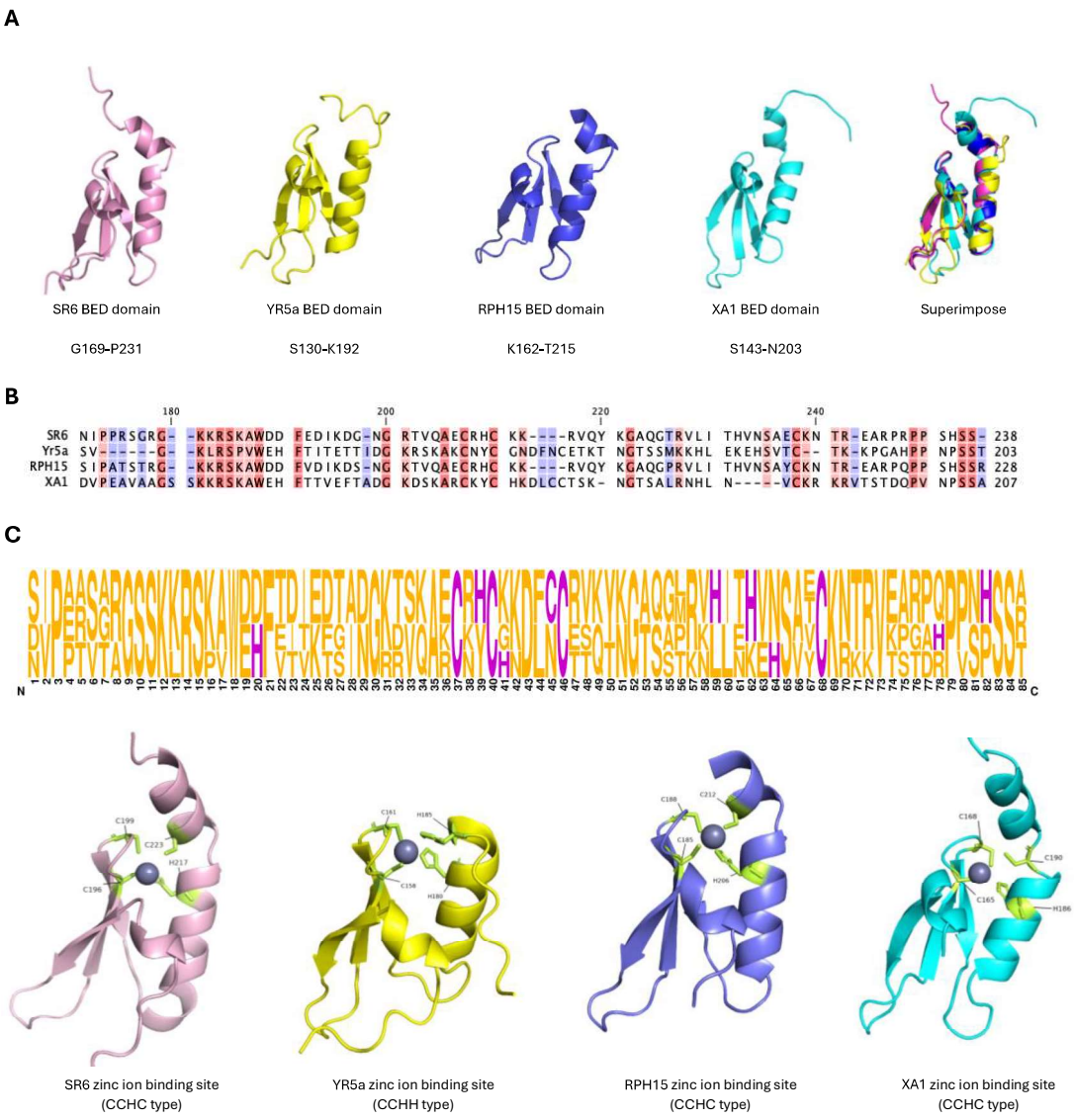


Supplementary Fig. 14. Comparison of the predicted structures of SR6, SR13, and SR21
A: Full length protein structures of SR6, SR13, and SR21 predicted by AlphaFold2. The three conserved subdomains are labelled in cyan (N terminal domains), salmon (NB-ARC domains), and limon (LRR domains); **B:** The distinct N termini of SR6, SR13, and SR21; **C:** The most conserved three helices of the three proteins are labelled in magenta and in order. The α3 helix of each protein is the closest α helix to the LRR domain (limon color).



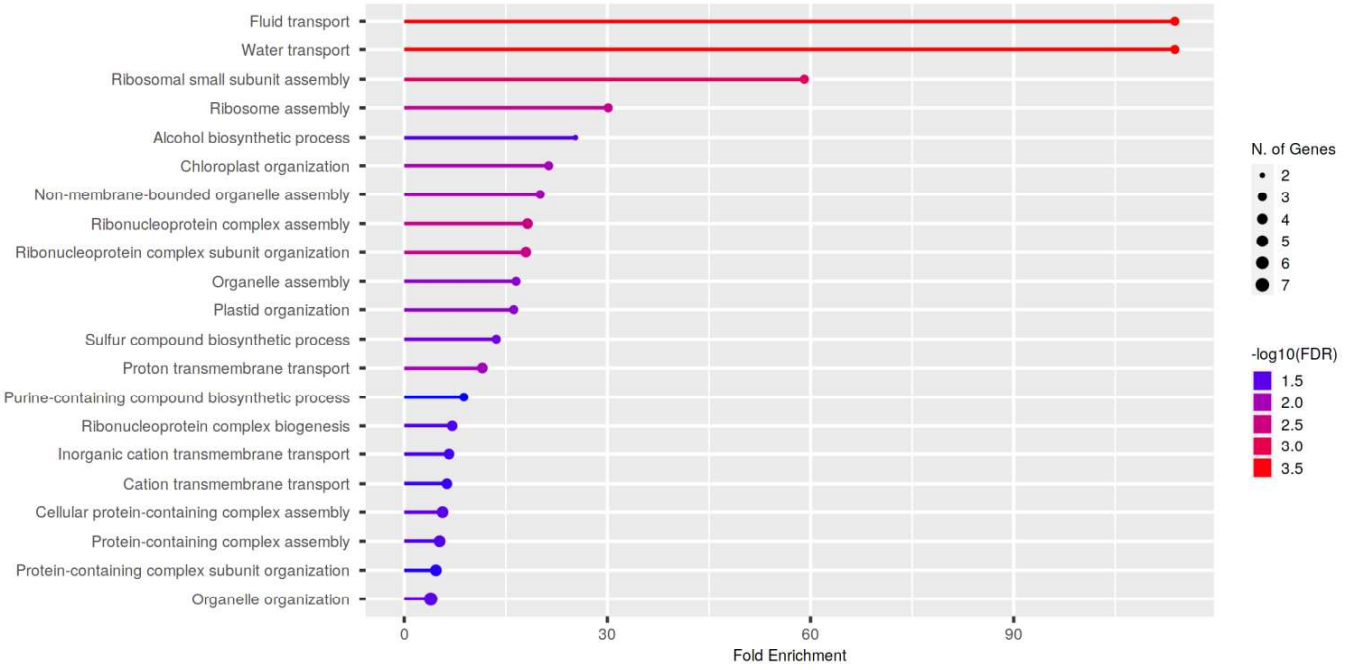
Supplementary Fig. 15. Full-length structures of SR6, SR13, and SR21 predicted by AlphaFold2 shaded with pLDDT values

The pLDDT (predicted Local Distance Difference Test) value of the three predicted full-length proteins are shaded with rainbow colours; red representing high pLDDT value (high confidence) and blue indicating a low pLDDT value (low confidence). The pLDDT values were calculated as ranging from 19.23 to 96.50 for SR6 (**A**), from 26.50 to 96.00 for SR13 (**B**), and 23.95 to 96.73 for SR21 (**C**) in a 0 to 100 scale.



Supplementary Fig. 16. Comparison of the predicted zinc finger BED domains and their zinc ion binding sites of SR6, YR5a, RPH15, and XA1

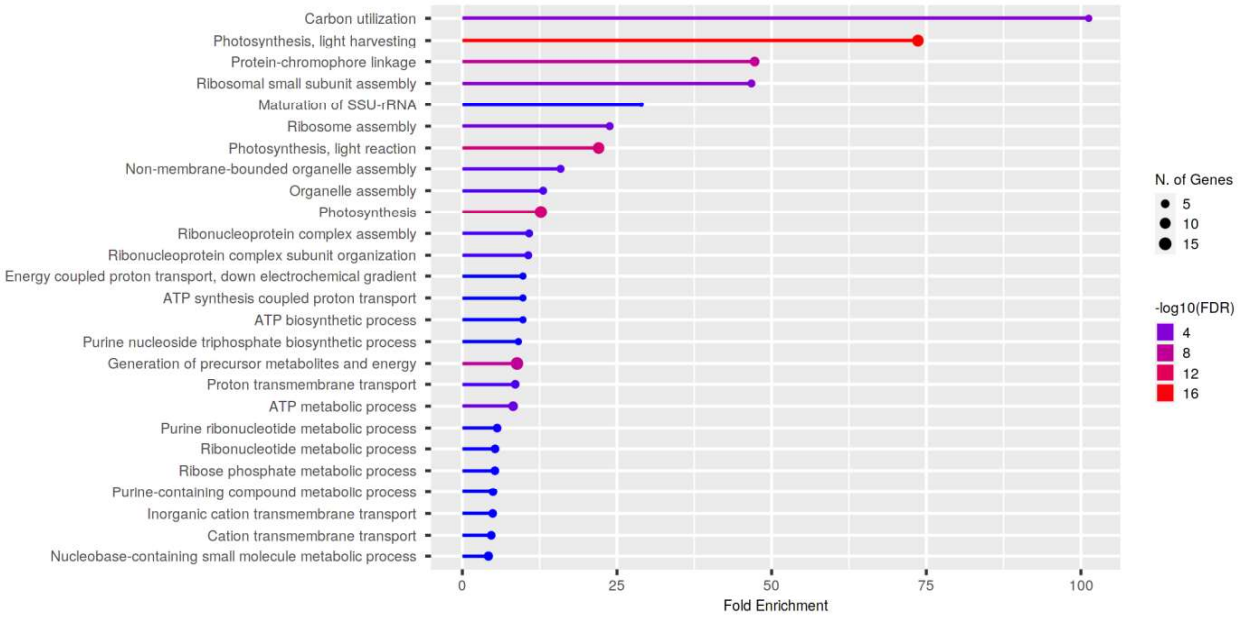
BED motifs from *Sr6*, wheat stripe rust resistance gene *Yr5*, barley leaf rust resistance gene *Rph15*, and rice bacterial blight resistance gene *Xa1* show high structural similarity (**A**) despite their low similarity in sequence alignment (**B**). **C:** WebLogo figure showing less conservation between the Zinc finger BED domains of SR6, YR5, RPH15, and XA1 with all the cysteines and histidines that are predicted to bind to zinc ions (Zn^{2+}) and resemble a "finger" highlighted in magenta (upper panel). AlphaFold3 predicted the potential zinc ion binding site in SR6, YR5a, RPH15, and XA1, indicating that there are two types of the zinc finger structures among these four proteins, CCHC type (SR6, RPH15, and XA1) and CCHH type (YR5a) (lower panel).



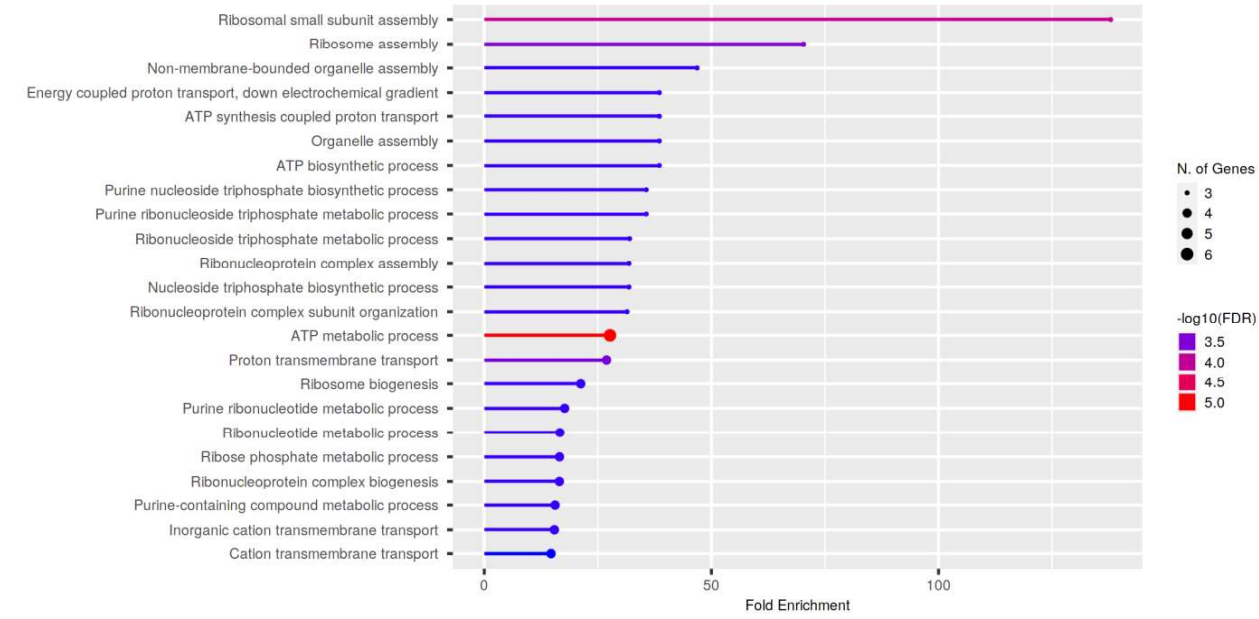
SR13CRD3 VS LMPGCRD3

Supplementary Fig. 17. Fold enrichment analysis of genes upregulated in LMPG+*Sr13* under low temperature. Round dots represent the number of genes with larger dots representing a higher number of genes. X axis represents the fold enrichment calculated based on the number of genes associated with the particular pathways/biological processes. Y axis represents pathway. Red color represents the most significant process and blue are the less significant in terms of -log10(FDR).

Note: No significant pathway was observed at Day 1, low temp.

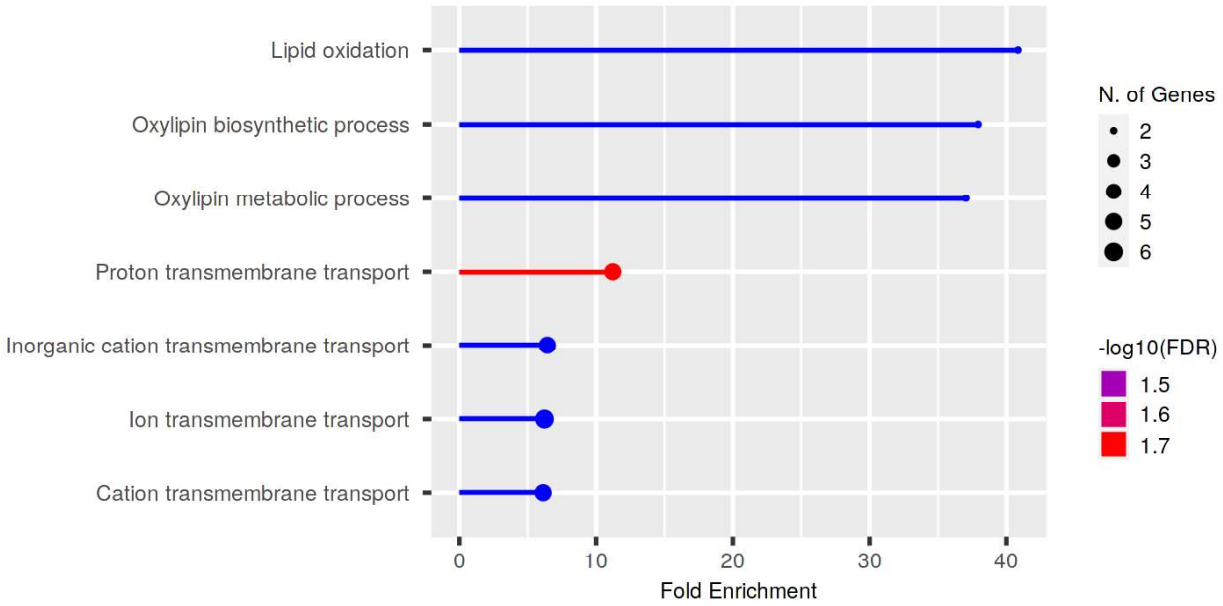


A: SR13HRD1 VS LMPGHRD1

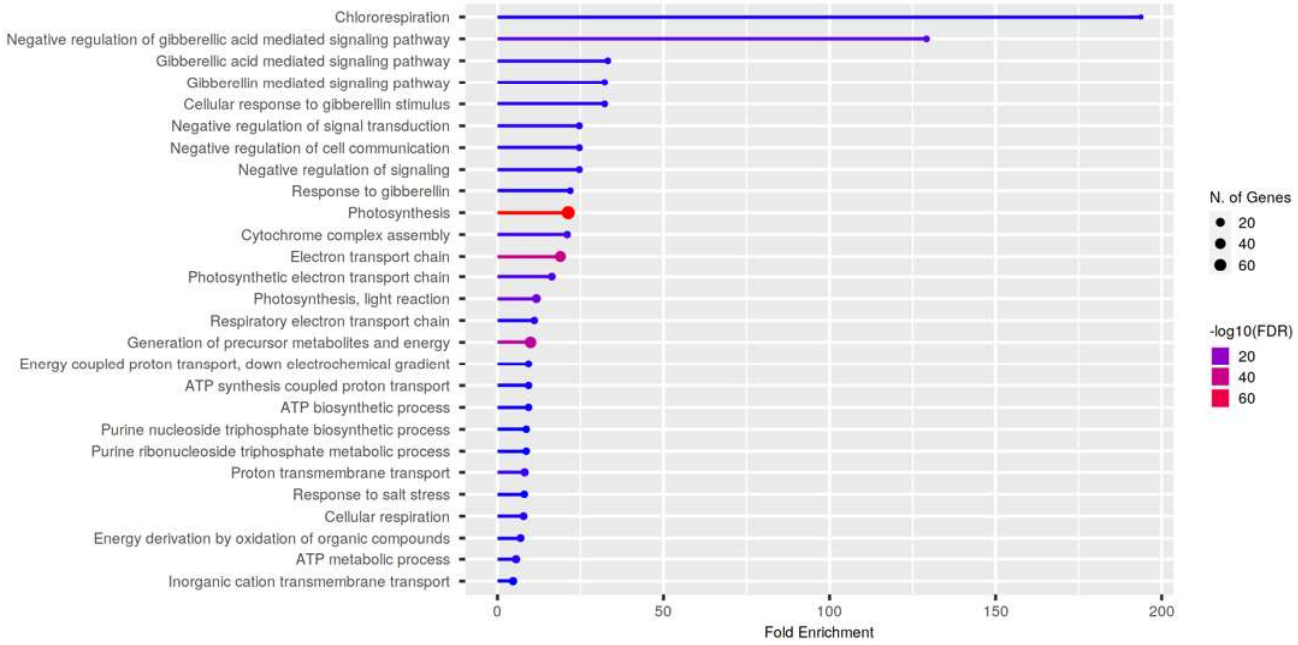


B: SR13HRD3 VS LMPGHRD3

Supplementary Fig. 18. Fold enrichment analysis of genes upregulated in LMPG+*Sr13* under high temperature. **A:** Genes/pathways expressed at Day 1, **B:** Genes/pathways expressed at Day 3. Round dots represent the number of genes where larger dots represent a higher number of genes. X axis represents the fold enrichment calculated based on the number of genes associated with the particular pathway/biological process. Y axis represents the pathway. Red color represents the most significant process and blue are less significant in terms of -log10(FDR).

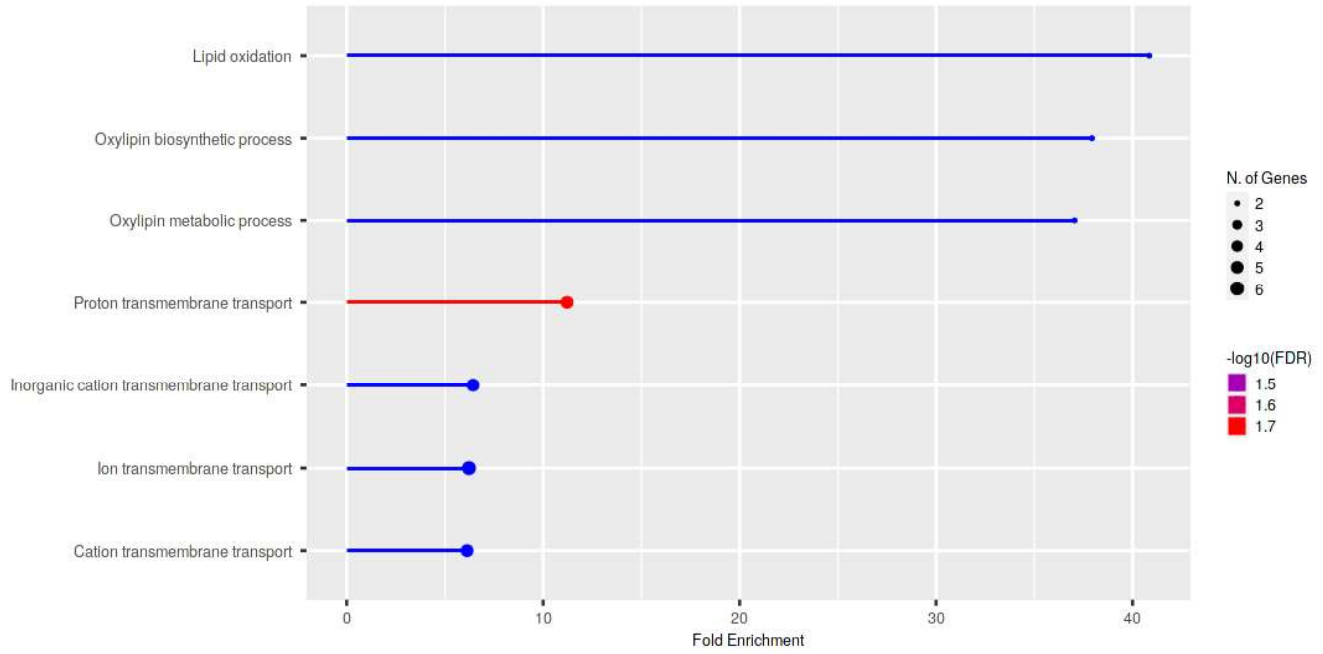


A: SR21CRD1 VS LMPGCRD1

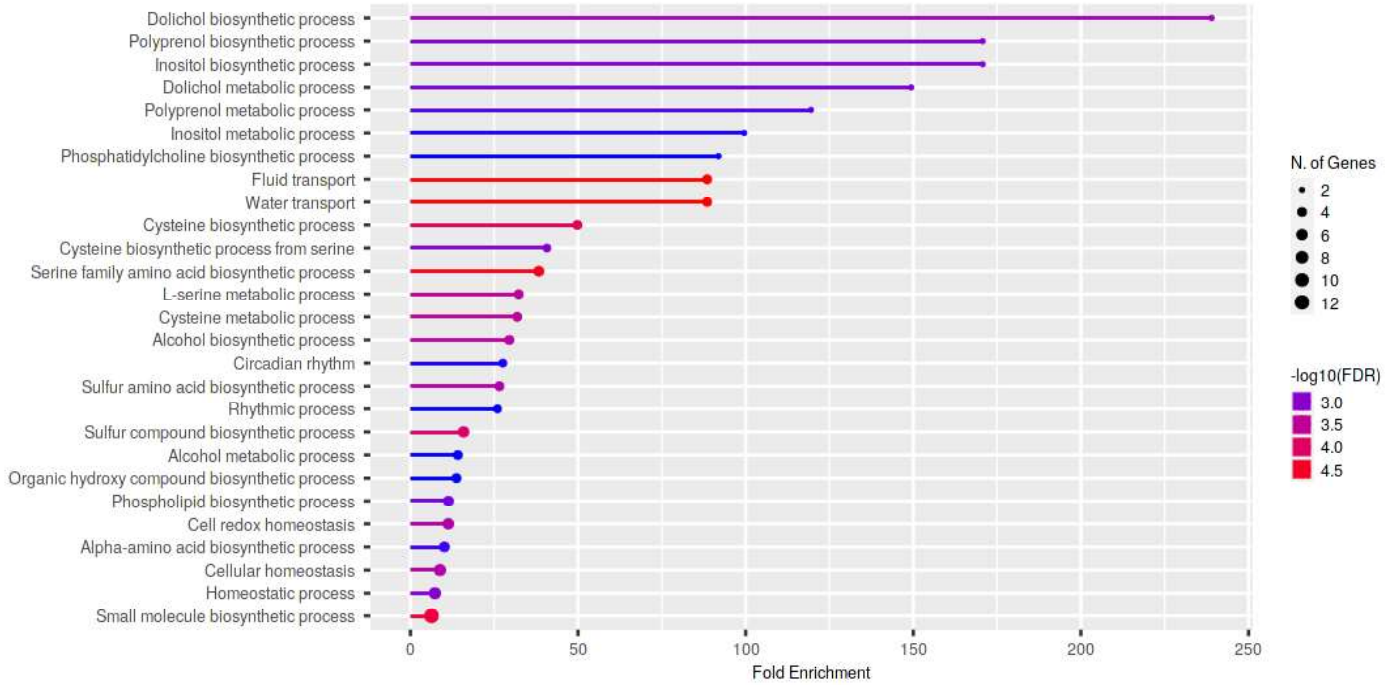


B: SR21CRD3 VS LMPGCRD3

Supplementary Fig. 19. Fold enrichment analysis of genes upregulated in LMPG+*Sr21* under low temperature. A: Genes/pathways expressed at Day 1; **B:** Genes/pathways expressed at Day 3. Round dots represent the number of genes where larger dots represent higher number of genes. X axis represents the fold enrichment calculated based on the number of genes associated to the pathway/biological process. Y axis represents pathway. Red color represents the most significant process and blue are less significant in terms of -log10(FDR).



A: SR21HRD1 VS LMPGHRD1



B: SR21HRD3 VS LMPGHRD3

Supplementary Fig. 20. Fold enrichment analysis of genes upregulated in LMPG+*Sr21* under high temperature. A: Genes/pathways expressed at Day 1; **B:** Genes/pathways expressed at Day 3. Round or bubble dot represents the number of genes where larger dots represent higher number of genes. X axis represents the fold enrichment calculated based on the number of genes associated to the particular pathway/biological process. Y axis represents pathway. Red color represents the most significant process and blue are the less significant interms of -log10(FDR).

Supplementary Table 1. Polymorphisms observed in point mutants of *Sr6*.

Line ID	Change in CDS	Change in protein	Exon/Intron	Predicted domain
3704-6	C1388T C4572T	P463L synonymous	Exon4 Exon4	NB-ARC between motif4 (Kin-2) and motif5 (RNBS-B)
3706-7	C4243T	L1415F	Exon4	LRR
3981-4	G(-)20A	Translation efficiency?	Exon1	5'UTR
4002-6	G1923T	L641F	Exon4	NB-ARC motif8 (Linker)
4190-6	G4309A	E1437K	Exon4	LRR
5047-4	G4466A G4507A	C1489Y E1503K	Exon4 Exon4	LRR LRR
5140-5	G3048A	W1016*	Exon4	LRR