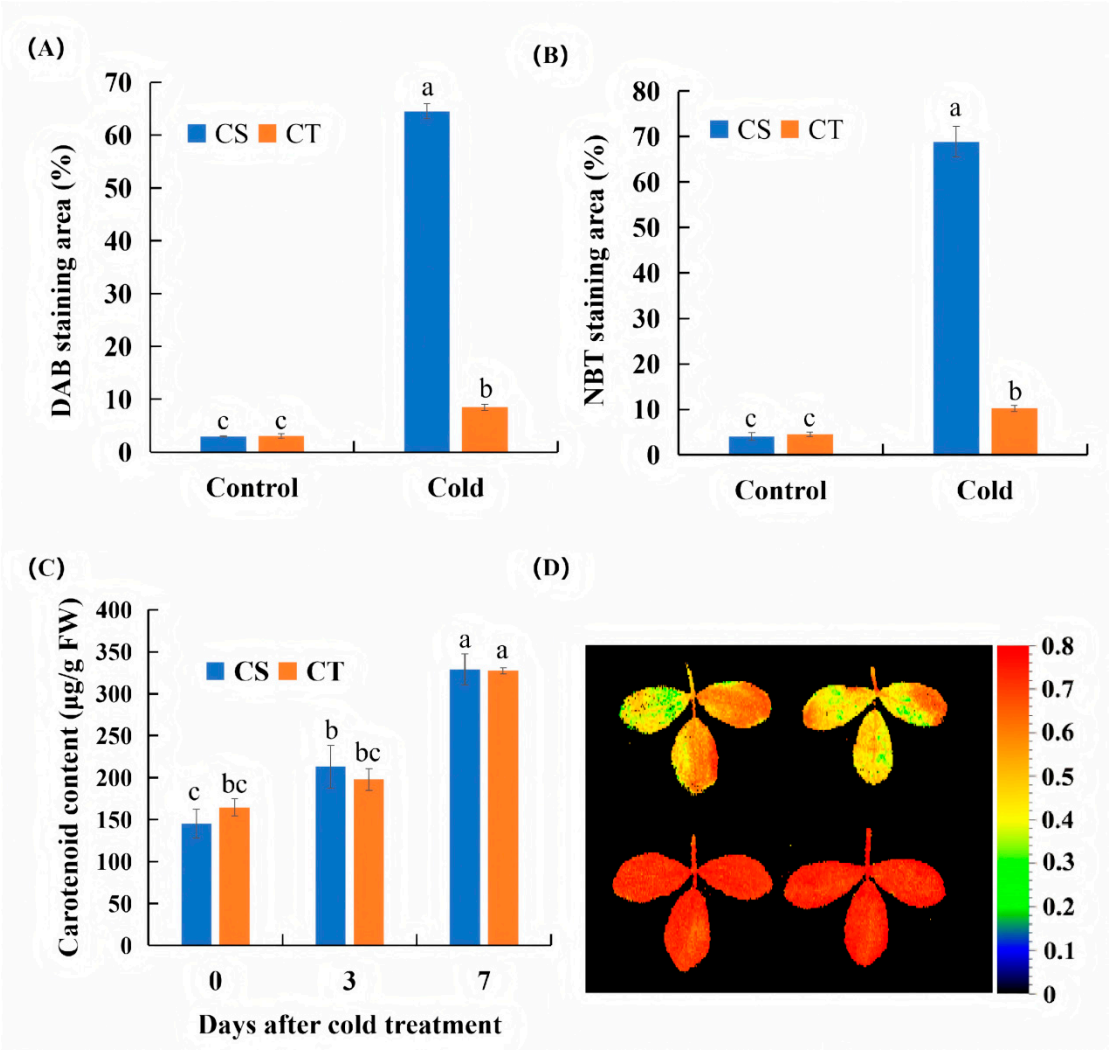


Figure S1. Quantitative analysis of ROS accumulation, carotenoid content, **and representative false-color image of PSII efficiency under cold stress.**

(A,B) Quantification of DAB staining for H_2O_2 (A) and NBT staining for $O_2^{\bullet-}$ accumulation (B) in leaves under control conditions (24°C) and after 7 days of cold treatment at 4°C. (C) Carotenoids contents in leaves under control conditions (24°C) and after 3 and 7 days of cold treatment (4°C). (D) Representative false-color image of the maximum photochemical efficiency of photosystem II (*Fv/Fm*) in leaves under control and cold stress conditions. Blue indicates lower *Fv/Fm* values, whereas red indicates higher *Fv/Fm* values. Scale bar = 1 cm. Values represent means ± SE of three biological replicates. Different lowercase letters indicate significant differences among treatments ($p < 0.05$).

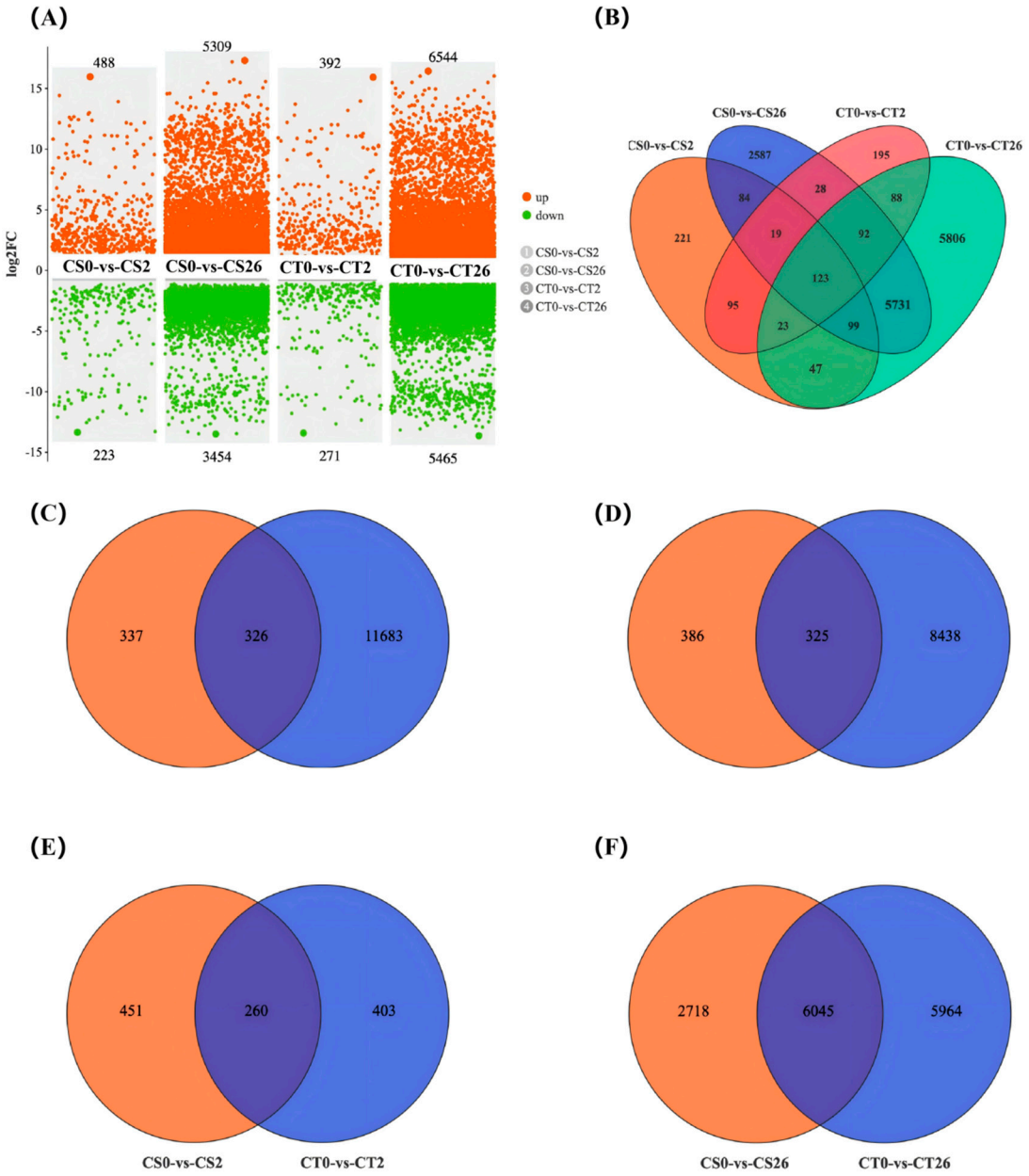


Figure S2. Comparative analysis of differentially expressed genes (DEGs) between cold-sensitive (CS) and cold-tolerant (CT) alfalfa cultivars under cold stress.

(A) Numbers of up- and down-regulated DEGs identified in CS and CT at 2 h and 26 h after cold treatment. (B) Venn diagram showing the overlap of DEGs among CS and CT at different time points. (C,D) Venn diagrams showing common and genotype-specific DEGs between early and prolonged cold responses within CS (C) and CT (D). (E,F) Venn diagrams showing common and genotype-specific DEGs between CS and CT at 2 h (E) and 26 h (F) after cold treatment. DEGs were identified using the criteria of $|\log_2(\text{fold change})| \geq 2$ and FDR ≤ 0.05. CS0 and CT0 represent control samples; CS2 and CS26 represent CS plants treated at 4°C for 2 h and 26 h, respectively; CT2 and CT26 represent CT plants treated at 4°C for 2 h and 26 h, respectively.

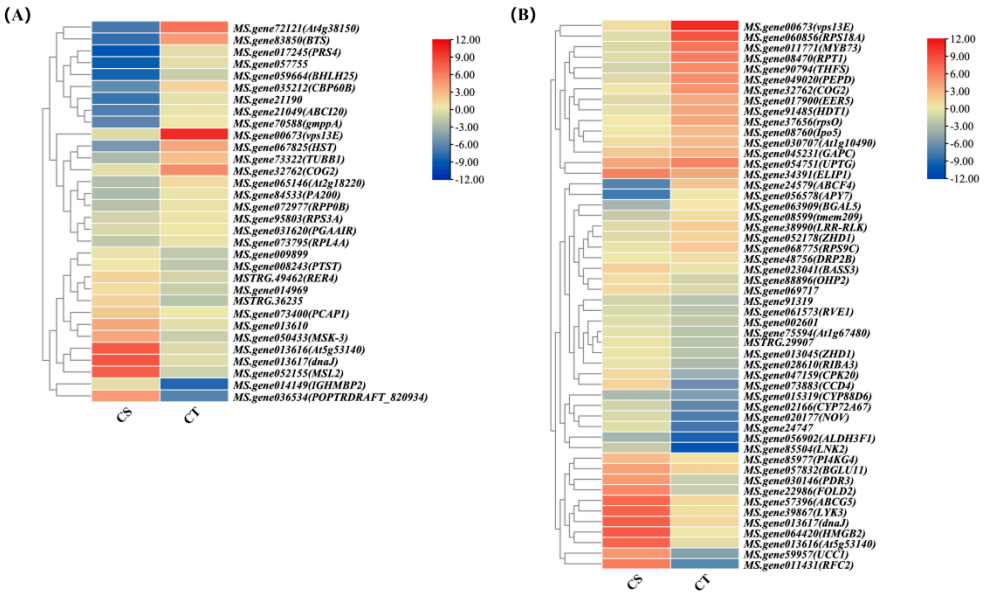


Figure S3. Expression changes of selected genes with significant cultivar-by-time interactions during cold exposure.

Heatmaps show DESeq2-estimated $\log_2$ fold changes relative to the respective 0 h baseline in the cold-sensitive (CS) and cold-tolerant (CT) cultivars at (A) 2 h (32 genes) and (B) 26 h (52 genes). Displayed genes had significant time-specific interactions (Benjamini–Hochberg-adjusted $p < 0.05$), remained significant under both sensitivity filters, and showed no comparison-relevant replicate-consistency flags. Entries with identical raw-count profiles were excluded from the display subsets. Colors indicate within-cultivar expression changes using a shared scale without row-wise standardization. Gene labels show gene IDs followed by symbols where available.

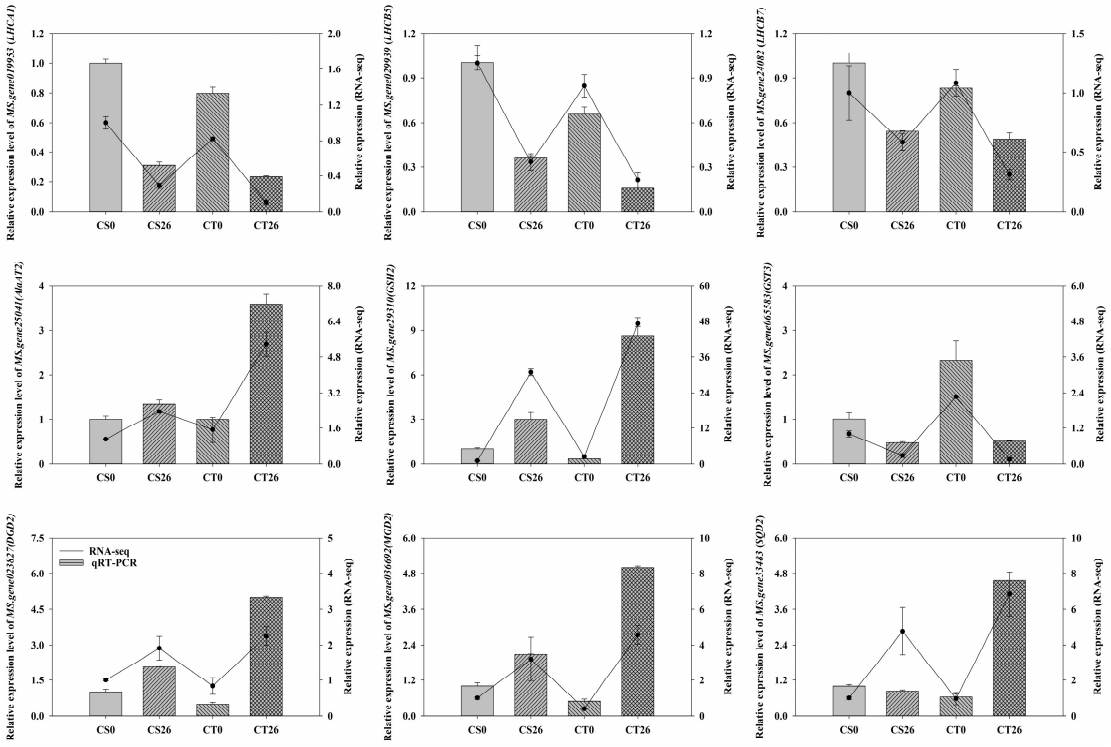


Figure S4. Comparison of relative expression levels of selected DEGs determined by qRT-PCR and RNA-seq in two alfalfa cultivars.

The relative expression levels of selected DEGs, including *LHCA1*, *LHCB5*, *LHCB7*, *AlaAT2*, *GSH2*, *GST3*, *DGD2*, *MGD2*, and *SQD2*, were determined by qRT-PCR using the $2^{-\Delta\Delta Ct}$ method, with MsACT7 as the internal reference gene. qRT-PCR-derived fold changes were compared with RNA-seq-derived FPKM values from the corresponding comparisons. Values represent means ± SE of three biological replicates.For RNA-seq, relative expression levels were calculated from normalized read counts. In both analyses, CS-0 was used as the common reference group, with its expression level set to 1. Relative expression levels were compared across four groups (CS0, CS26, CT0, and CT26) to assess the consistency of expression patterns between the two methods. Values represent means ± SE of three biological replicates.

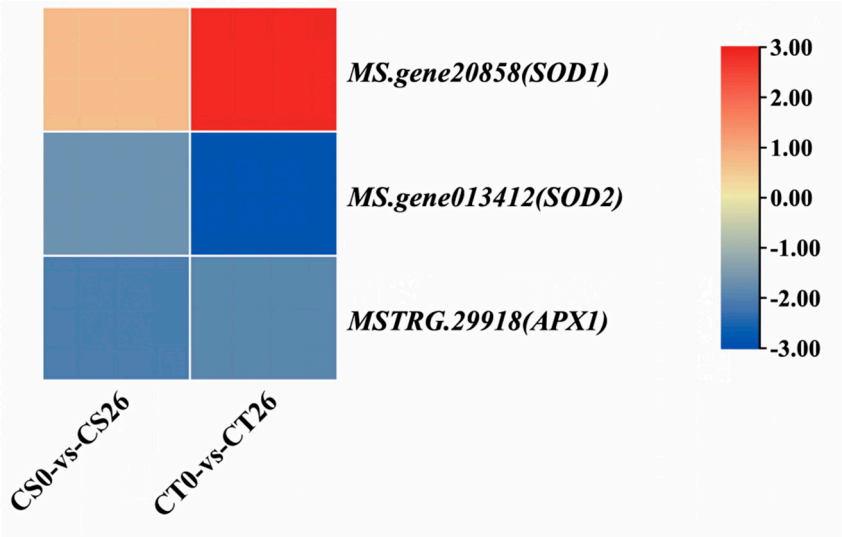


Figure S5. Expression patterns of SOD1, SOD2, and APX1 under cold stress. Heatmap showing the expression patterns of SOD1, SOD2, and APX1 in the comparisons CS0 vs. CS26 and CT0 vs. CT26. The color scale represents $\log_2$(Fold Change) values, with blue indicating lower expression levels and red indicating higher expression levels.