

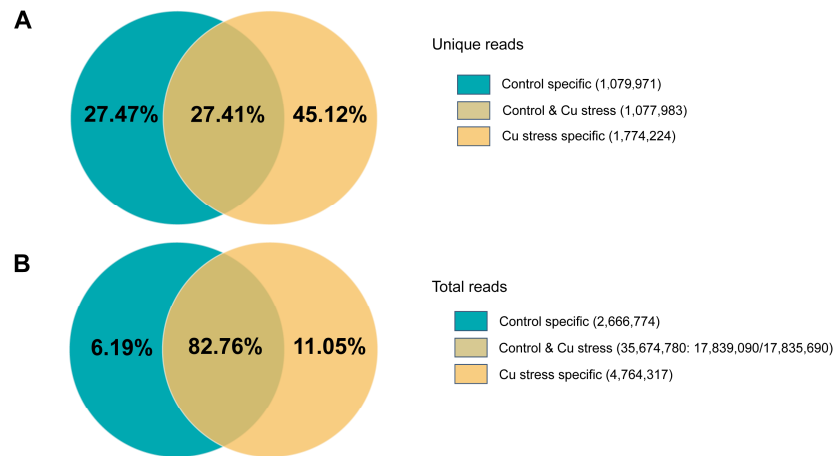


**Figure S1.** Venn diagrams illustrating unique (A) and total (B) reads identified in the control and Cu stress libraries from *Helianthus tuberosus*. Percentages and numbers in parentheses indicate the proportion and the specific or shared quantities of reads in the two libraries, respectively.

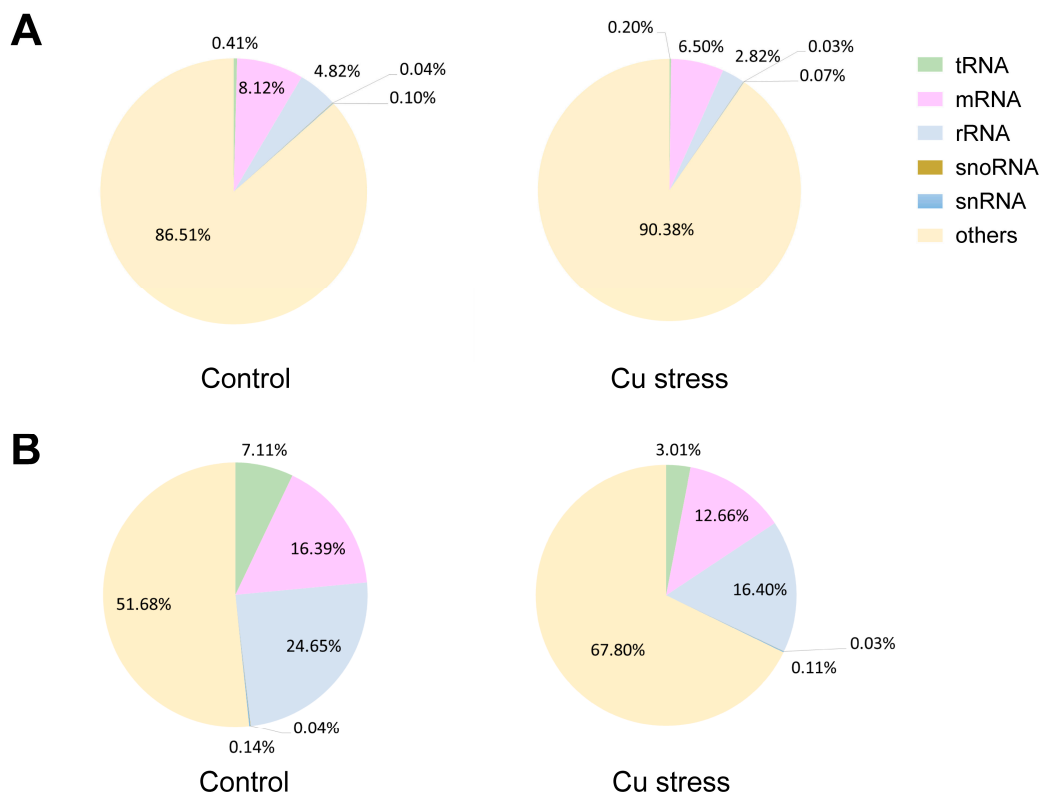


**Figure S2.** Distribution of unique (A) and total (B) read categories in the two *Helianthus tuberosus* libraries.

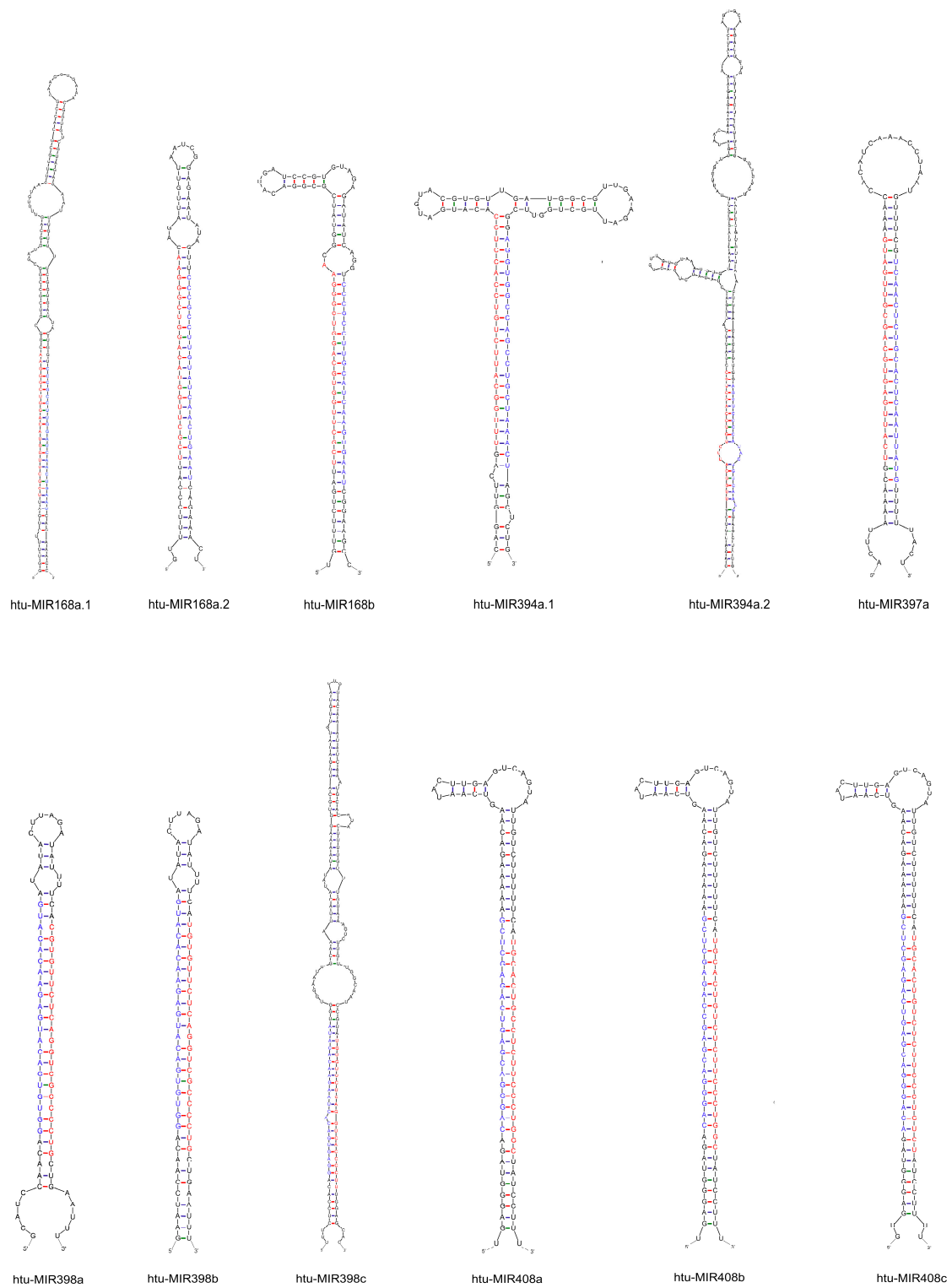


**Figure S3.** Predicted stem-loop structures of MIR168, MIR394, MIR397, MIR398, and MIR408 in *Helianthus tuberosus*.

The sequences of miRNA and miRNA\* are marked in red and blue, respectively.

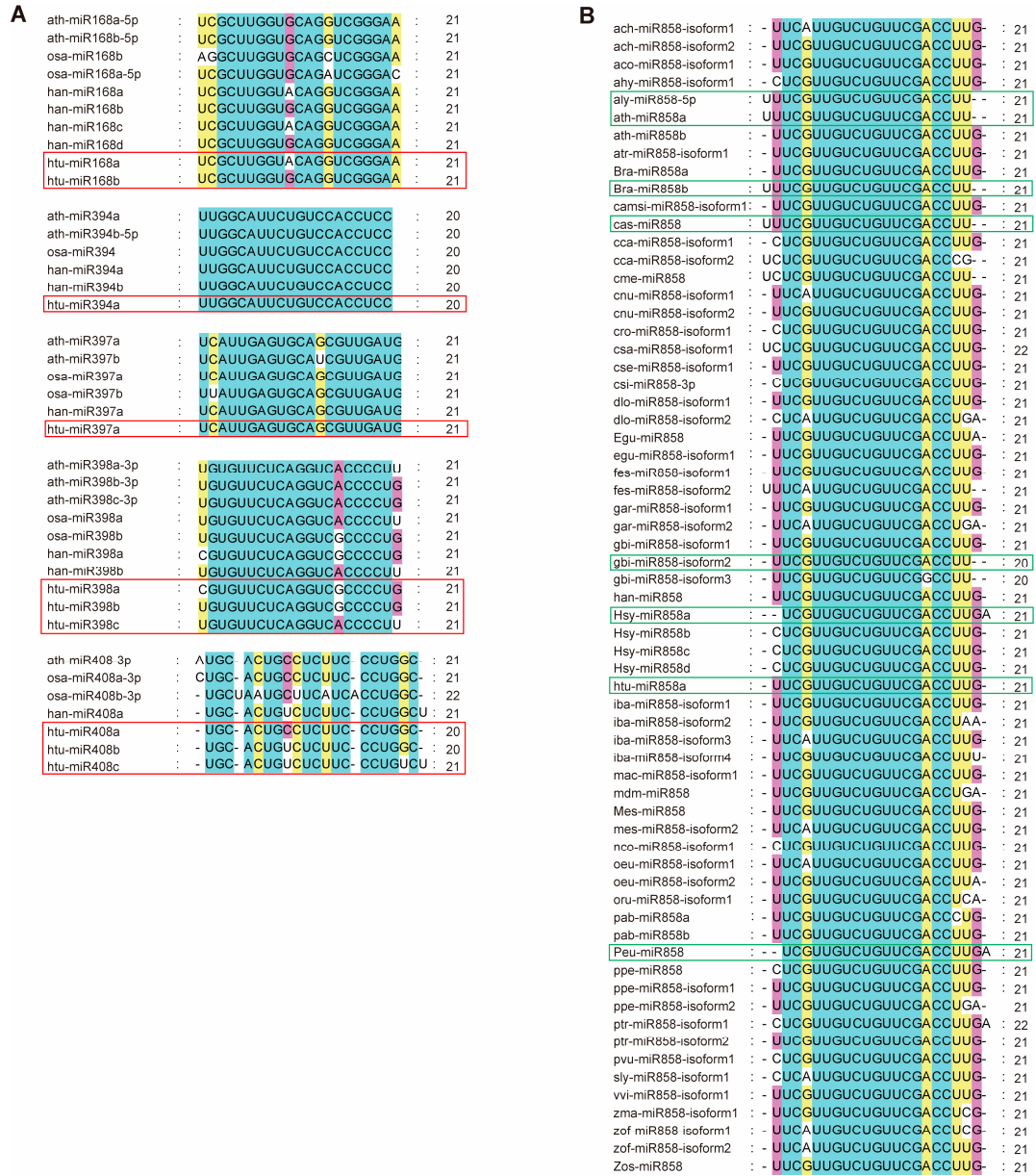


**Figure S4.** Multiple sequence alignment of six miRNA family members across various plant species. (A) Alignment of miR168, miR394, miR397, miR398, and miR408 from *Arabidopsis thaliana* (Ath), *Oryza sativa* (Osa), *Helianthus annuus* (Han), and *Helianthus tuberosus* (Htu). (B) Alignment of miR858 from various plant species retrieved from miRBase, PmiREN, and Wang et al. (2023) (see Supplementary Table 3). The red rectangle indicates miRNA sequences, while the green box highlights unique miR858 isoforms identified in *Helianthus tuberosus* in this study. Alignment was conducted using MEGA7 and visualized with GENEDOC.

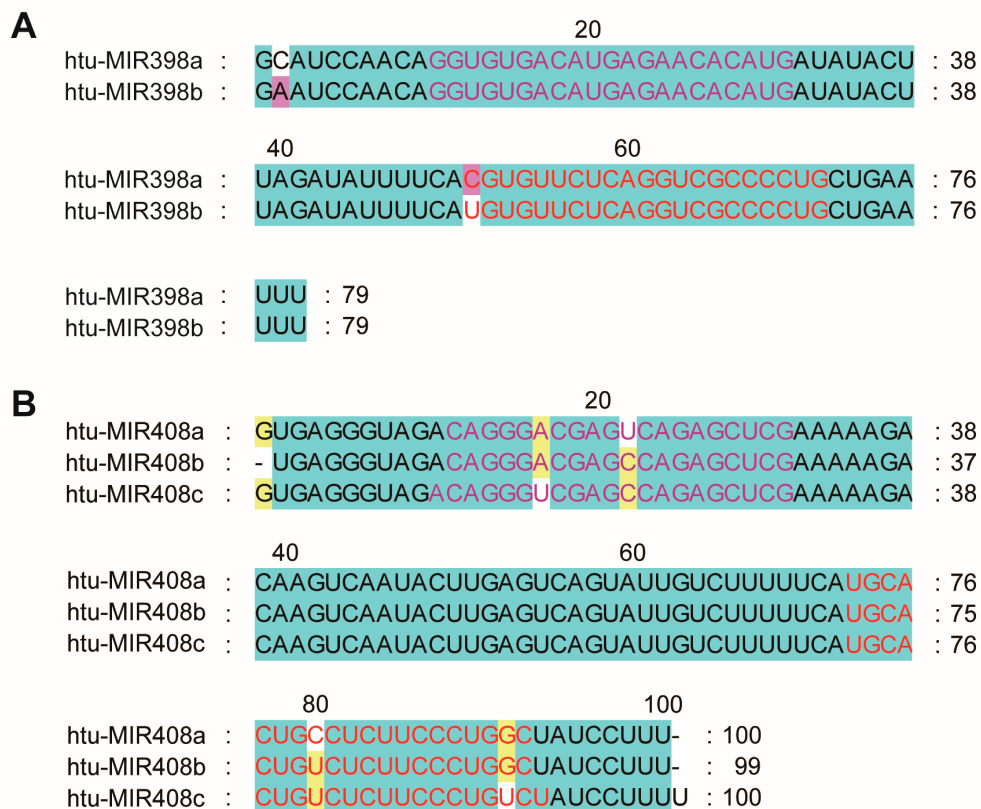


**Figure S5.** Alignment of the precursors of miR398 (A) and miR408 (B) in *Helianthus tuberosus*. The sequences of miRNA and miRNA\* are highlighted in red and purple, respectively.

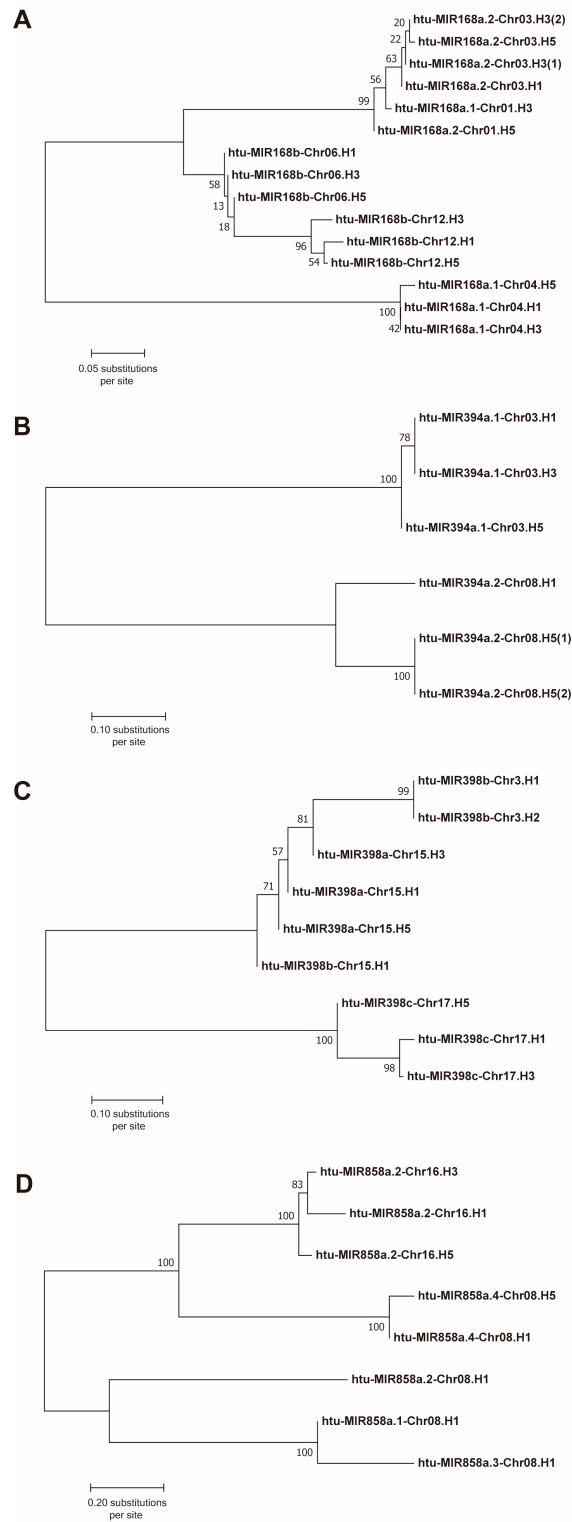


**Figure S6.** Phylogenetic trees of the precursors of miR168, miR394, miR398, and miR858 in *Helianthus tuberosus*.

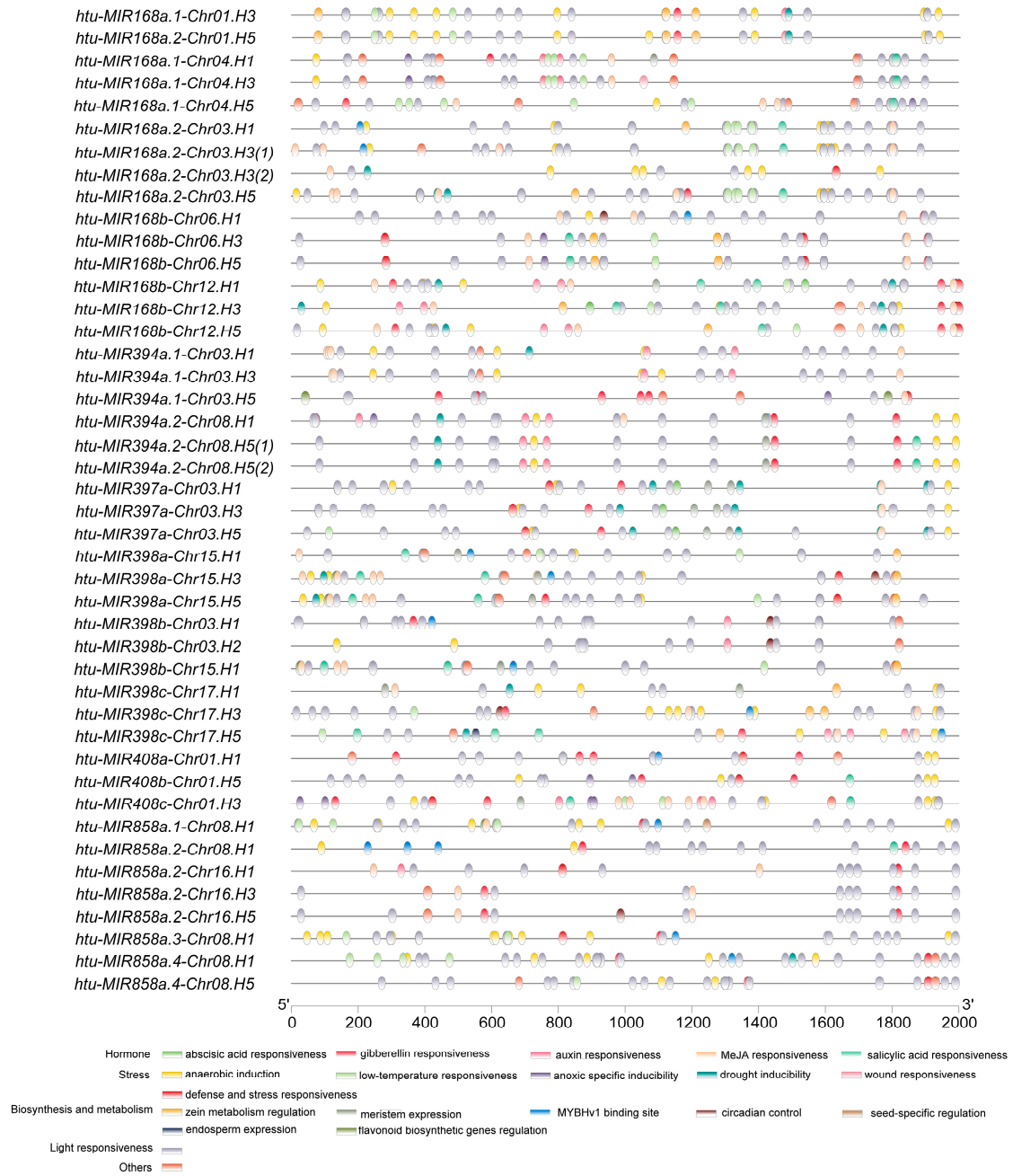


**Figure S7.** The predicted *cis*-acting elements in the promoters of *MIRNA* genes in *Helianthus tuberosus*.

The 2.0 kb upstream regions of the *MIRNA* genes were analyzed and predicted using the PlantCARE database and subsequently visualized using TBtools.

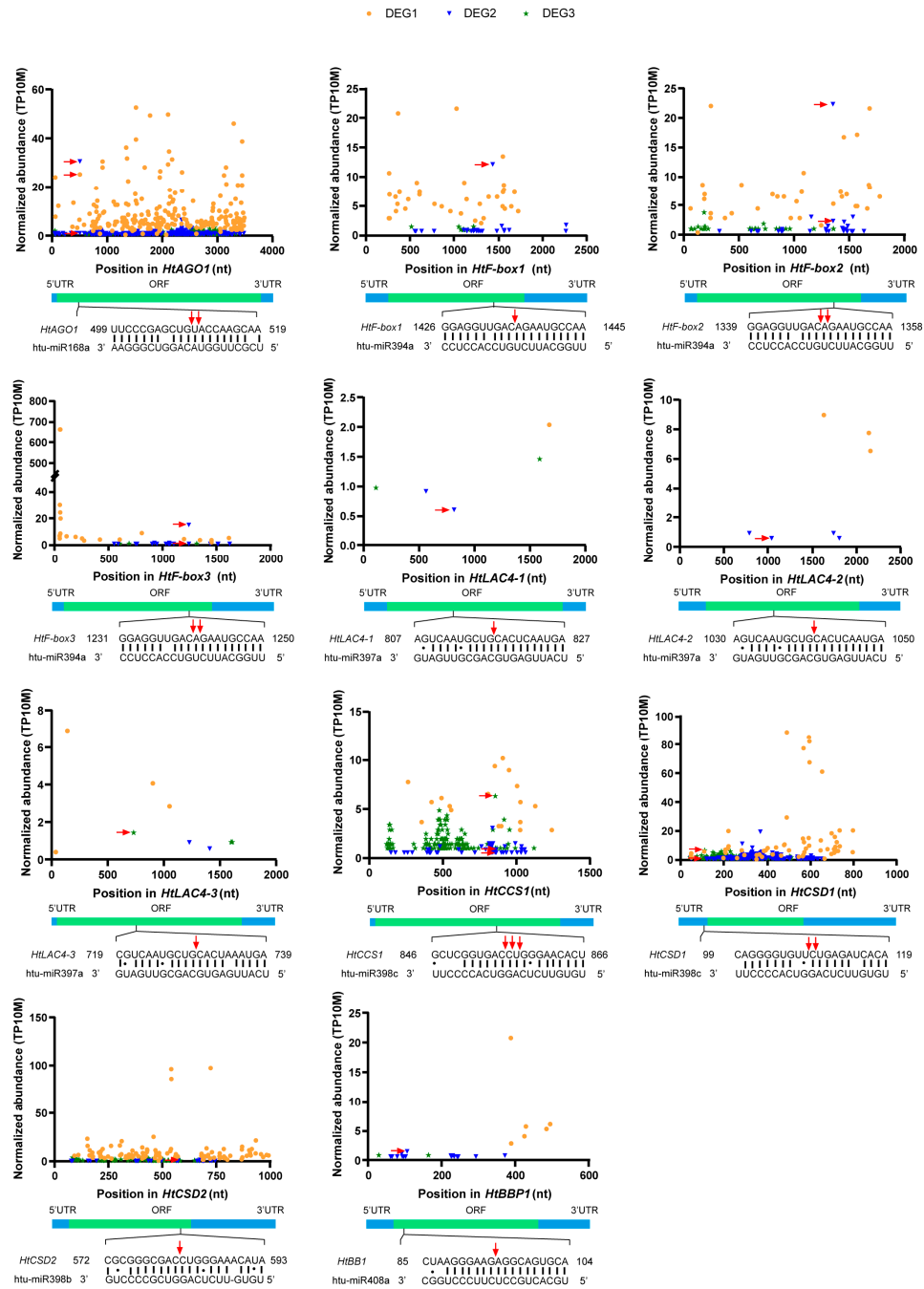


**Figure S8.** t-plots of the targets of the miR168, miR394, miR397, miR398, and miR408 identified from three degradome libraries in *Helianthus tuberosus*.

Eleven genes were targeted by miRNA. Refer to Fig. 5 for additional information.



**Figure S9.** Multiple sequence alignment of fifteen *HtMyb* genes targeted by htu-miR858, showing 50 bases from the 9th position of the miR858 binding site (5'→3').

**Table S1.** Length distribution of unique and total reads in two libraries of *Helianthus tuberosus*.

| Length (nt) | CK-     | %     | CK-total | %     | Cu-unique | %     | Cu-total | %      |
|-------------|---------|-------|----------|-------|-----------|-------|----------|--------|
| All         | 2157954 | 100.0 | 20505864 | 100.0 | 2852207   | 100.0 | 22600007 | 100.00 |
| 19          | 31936   | 1.48  | 636282   | 3.10  | 38071     | 1.33  | 572722   | 2.53   |
| 20          | 44434   | 2.06  | 658008   | 3.21  | 58785     | 2.06  | 696100   | 3.08   |
| 21          | 246064  | 11.40 | 5261541  | 25.66 | 321408    | 11.27 | 6182004  | 27.35  |
| 22          | 149618  | 6.93  | 1603850  | 7.82  | 208132    | 7.30  | 1954872  | 8.65   |
| 23          | 279535  | 12.95 | 1368756  | 6.67  | 429327    | 15.05 | 1958217  | 8.66   |
| 24          | 1188635 | 55.08 | 5113354  | 24.94 | 1603275   | 56.21 | 7538708  | 33.36  |
| 25          | 33809   | 1.57  | 338451   | 1.65  | 51327     | 1.80  | 351702   | 1.56   |
| Sum         | 1974031 | 91.48 | 14980242 | 73.05 | 2710325   | 95.03 | 19254325 | 85.20  |

**Table S2.** Sequence of miRNA\* corresponding to six Cu-responsive miRNA families in *Helianthus tuberosus*.

| miRNA member   | Sequence               | Length<br>(nt) | Control<br>reads | Cu-stress<br>reads |
|----------------|------------------------|----------------|------------------|--------------------|
| htu-miR168a.1* | CCCGCCTTGGATCAACTGAAT  | 21             | 104              | 78                 |
| htu-miR168a.2* | CCCGCCTTGTATCAACTGAAT  | 21             | 270              | 153                |
| htu-miR168b*   | CCCGCCTTGCATCAAGTGAAT  | 21             | 567              | 622                |
| htu-miR394a.1* | AGGTGGCCAGCCTGCTAAACT  | 21             | 5                | 0                  |
| htu-miR394a.2* | AGGTGGGCATACTGTCAATG   | 20             | 22               | 32                 |
| htu-miR397a*   | TCAACTCTGCACTCAATTATG  | 21             | 2132             | 406                |
| htu-miR398a*   | GGTGTGACATGAGAACACATG  | 21             | 74               | 8                  |
| htu-miR398b*   | GGTGTGACATGAGAACACATG  | 21             | 74               | 8                  |
| htu-miR398c*   | GGAGTGACACTAAGAACACATA | 22             | 0                | 5                  |
| htu-miR408a*   | CAGGGACGAGTCAGAGCTCG   | 20             | 2                | 0                  |
| htu-miR408b*   | CAGGGACGAGCCAGAGCTCG   | 20             | 0                | 0                  |
| htu-miR408c*   | ACAGGGTCGAGCCAGAGCTCG  | 21             | 69               | 0                  |
| htu-miR858a.1* | AGGTGGAACAGTCAATGAATG  | 21             | 0                | 0                  |
| htu-miR858a.2* | AGGTGGAACAGACGATGAATG  | 21             | 12               | 0                  |
| htu-miR858a.3* | AGGTGGAACAGACAATGAATG  | 21             | 0                | 0                  |
| htu-miR858a.4* | AGGTGGGACAGACAATGAATG  | 21             | 1                | 0                  |

**Table S7.** Properties analysis of targets from six miRNA families in *Helianthus tuberosus*. AA: Amino acids; MW: Molecular weight; pI: Isoelectric point; GRAVY: Grand average of hydropathicity.

| NO | Gene name       | Accession number | miRNA  | Category of targets | CDS length (bp) | Number of AA | MW (kDa) | pI   | GRAVY  | Subcellular localization |
|----|-----------------|------------------|--------|---------------------|-----------------|--------------|----------|------|--------|--------------------------|
| 1  | <i>HtAGO1</i>   | PQ368255         | miR168 | I                   | 3177            | 1058         | 117.49   | 9.43 | −0.571 | nucleus                  |
| 2  | <i>HtF-box1</i> | PQ368256         | miR394 | I                   | 1320            | 439          | 49.63    | 9.05 | −0.052 | cytoplasm                |
| 3  | <i>HtF-box2</i> | PQ368257         | miR394 | I                   | 1401            | 466          | 53.41    | 9.04 | −0.244 | peroxisome               |
| 4  | <i>HtF-box3</i> | PQ368258         | miR394 | I                   | 1359            | 452          | 51.05    | 8.58 | −0.210 | endoplasmic reticulum    |
| 5  | <i>HtLAC4-1</i> | PQ368259         | miR397 | II                  | 1680            | 559          | 61.30    | 9.28 | 0.010  | chloroplast              |
| 6  | <i>HtLAC4-2</i> | PQ368260         | miR397 | II                  | 1689            | 562          | 61.72    | 9.21 | 0.044  | chloroplast              |
| 7  | <i>HtLAC4-3</i> | PQ368261         | miR397 | I                   | 1668            | 555          | 60.81    | 9.33 | −0.040 | chloroplast              |
| 8  | <i>HtCCS1</i>   | PQ368262         | miR398 | I                   | 948             | 315          | 33.44    | 6.14 | −0.100 | chloroplast              |
| 9  | <i>HtCSD1</i>   | PQ368263         | miR398 | I                   | 462             | 153          | 15.43    | 5.61 | −0.154 | cytoplasm                |
| 10 | <i>HtCSD2</i>   | PQ368264         | miR398 | III                 | 534             | 177          | 18.25    | 6.33 | −0.063 | chloroplast              |
| 11 | <i>HtBBP1</i>   | PQ368265         | miR408 | I                   | 381             | 126          | 13.12    | 9.68 | 0.183  | extracellular            |
| 12 | <i>HtMYB1</i>   | PQ368266         | miR858 | I                   | 723             | 240          | 27.22    | 5.45 | −0.875 | nucleus                  |
| 13 | <i>HtMYB2</i>   | PQ368267         | miR858 | I                   | 822             | 273          | 30.94    | 5.03 | −0.736 | nucleus                  |
| 14 | <i>HtMYB3</i>   | PQ368268         | miR858 | I                   | 957             | 318          | 34.46    | 5.66 | −0.453 | nucleus                  |
| 15 | <i>HtMYB4</i>   | PQ368269         | miR858 | I                   | 939             | 312          | 35.32    | 4.84 | −0.579 | nucleus                  |
| 16 | <i>HtMYB5</i>   | PQ368270         | miR858 | I                   | 765             | 254          | 29.33    | 8.96 | −0.912 | nucleus                  |
| 17 | <i>HtMYB6</i>   | PQ368271         | miR858 | I                   | 819             | 272          | 31.25    | 6.20 | −0.797 | nucleus                  |

| NO | Gene name      | Accession number | miRNA  | Category of targets | CDS length (bp) | Number of AA | MW (kDa) | pI   | GRAVY  | Subcellular localization |
|----|----------------|------------------|--------|---------------------|-----------------|--------------|----------|------|--------|--------------------------|
| 18 | <i>HtMYB7</i>  | PQ368272         | miR858 | I                   | 912             | 303          | 33.73    | 5.39 | −0.686 | nucleus                  |
| 19 | <i>HtMYB8</i>  | PQ368273         | miR858 | I                   | 762             | 253          | 86.71    | 4.99 | 0.832  | nucleus                  |
| 20 | <i>HtMYB9</i>  | PQ368274         | miR858 | I                   | 768             | 255          | 95.75    | 5.07 | 0.783  | nucleus                  |
| 21 | <i>HtMYB10</i> | PQ368275         | miR858 | I                   | 783             | 260          | 84.01    | 5.10 | 0.770  | nucleus                  |
| 22 | <i>HtMYB11</i> | PQ368276         | miR858 | I                   | 750             | 249          | 163.63   | 4.92 | 0.731  | nucleus                  |
| 23 | <i>HtMYB12</i> | PQ368277         | miR858 | I                   | 744             | 247          | 93.87    | 5.07 | 0.772  | nucleus                  |
| 24 | <i>HtMYB13</i> | PQ368278         | miR858 | I                   | 768             | 255          | 92.51    | 5.06 | 0.800  | nucleus                  |
| 25 | <i>HtMYB14</i> | PQ368279         | miR858 | I                   | 948             | 315          | 139.70   | 5.06 | 0.650  | nucleus                  |
| 26 | <i>HtMYB15</i> | PQ368280         | miR858 | I                   | 852             | 283          | 106.18   | 5.05 | 0.759  | nucleus                  |