



Genome-Wide Identification and Characterization of *TAS1/2*-like Loci Associated with the miR173–*TAS1/2* Regulatory Pathway in *Camelina sativa*

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Abstract

Trans-acting small interfering RNAs (tasiRNAs) produced from *TAS1/2* loci regulate *HEAT-INDUCED TAS1 TARGET* transcripts and mRNAs encoding pentatricopeptide repeat proteins in *Arabidopsis thaliana* (*A. thaliana*). However, their genomic organization in polyploid crops is unknown. Here, we identified and characterized *TAS1/2*-like loci (*TASIL/2L*) in 12 cultivars of the allohexaploid crop *Camelina sativa* (*C. sativa*) using conserved miR173/*TAS1c* 3'D6(–) trigger sites with *TAS1/2*-derived tasiRNAs. Through phasing analysis with publicly available sRNA-Seq dataset from one of 12 cultivars, we confirmed that these loci tend to produce small RNAs in a phased manner. We also identified that *TAS2–TAS1* arrangement in *A. thaliana* is also conserved in all identified *TASIL/2L* loci across the genome of 12 *C. sativa*'s cultivars. Furthermore, for *C. sativa* cv. Ames1043, identified *TAS2L–TASIL* loci showed a distinct miR173-triggered 21-nucleotide-length phasing, whereas one *TAS2L–TASIL* with a disrupted miR173-binding site exhibited weak aberrant phasing. Our findings suggest that the copy number variations in *TASIL*-derived tasiR255-like and *TAS1c* 3'D6(–)-like elements may contribute to cultivar-specific differences in the potential regulation of their target genes. Together, our results suggest a significant conservation of *TASIL/2L* and their relative genomic organization of *TAS1/2* loci across *C. sativa* cultivars and provide functioning of miR173-triggered phasing mechanism on *TASIL/2L*s, offering a concise framework for dissecting small-RNA regulatory networks in polyploid crops.

Keywords miR173 · *TAS1/2* · Trans-acting siRNA · *HEAT-INDUCED TAS1 TARGET* · *Camelina sativa*

Abbreviations

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HTT	HEAT-INDUCED TAS1 TARGET
miR173	microRNA 173
PPR	Pentatricopeptide repeat
siRNA	Small interfering RNA
sRNA	Small RNA
sRNA-seq	Small-RNA sequencing
tasiRNA	Trans-acting small interfering RNA
<i>TASIL/2L</i>	<i>TAS1/2</i> -like locus
<i>TASIL</i>	<i>TAS1</i> -like locus
<i>TAS2L</i>	<i>TAS2</i> -like locus

Introduction

Trans-acting small interfering RNA (tasiRNA) is a class of phased siRNA that plays an essential role via post-transcriptional gene silencing in plant. The biogenesis of tasiRNA is known to be initiated by a precursor RNA cleavage mediated by microRNA (miRNA) or other siRNAs, defining the register for subsequent processing (Allen et al. 2005). One of the resulting fragments is stabilized, potentially with the aid of SUPPRESSOR OF GENE SILENCING 3, and converted into double-stranded RNA by RNA-dependent RNA polymerase 6 (Vazquez et al. 2004; Yoshikawa et al. 2005). This double-stranded RNA is then processed by DICER-LIKE 4 into 21-nucleotide (nt) siRNA duplexes with 2 nt 3'-overhangs. Finally, one strand of each duplex is incorporated into ARGONAUTE proteins (Mi et al. 2008; Montgomery et al. 2008; Takeda et al. 2008), which can target specific transcripts for cleavage or translational repression. These mechanisms play a pivotal role in regulating biological processes such as leaf development and stress responses in plant (Chen 2009; Ruiz-Ferrer and Voinnet 2009; Katiyar-Agarwal and Jin 2010).

Within this tasiRNA pathway, eight tasiRNA-generating genes have been characterized in *Arabidopsis thaliana* (*A. thaliana*) and are grouped into four families (*TASI-4*) (Chen 2009). Among these, the *TASI* and *TAS2* gene families represent well-characterized models of phased small RNA biogenesis initiated by miR173. *TASI/2*-derived tasiRNAs predominantly target mRNAs encoding pentatricopeptide repeat (PPR) proteins—a rapidly expanding clade known for its role in RNA processing. In addition, *TASI*-derived tasiRNAs target *HEAT-INDUCED TASI TARGET* (*HTT*) mRNAs, which are crucial for thermotolerance and stress adaptation (Yoshikawa et al. 2005; Howell et al. 2007; Li et al. 2014). Several phased siRNA-producing pathways are broadly conserved across land plants (Liu et al. 2020). However, the miR173–*TASI/2* system, an evolutionary descendant of the miR7122–*TASL*–*PPR* pathway (Xia et al. 2013), has been identified and studied mainly in *A. thaliana* and remains largely unexplored throughout viridiplantae (Axtell et al. 2006; Xia et al. 2013, 2017). This gap in our knowledge starkly calls for further investigation into their divergence and dynamic regulatory roles of *TASI/2*-homologous loci across diverse plant lineages.

To address this research gap, we have focused on *Camelina sativa* (*C. sativa*), an allohexaploid oilseed crop (2n=40) which provides a compelling system to examine tasiRNA regulatory mechanisms in a polyploid context. The genome of *C. sativa* is supposed to be derived from the hybridization of *Camelina neglecta* (n=6), *Camelina hispida* (n=7), and an unidentified subgenome (n=7) (Mandakova et al. 2019). It is characterized by extensive gene duplication and

regulatory plasticity, i.e., features that are likely to foster novel regulatory interactions. Beyond its genomic complexity, *C. sativa* exhibits promising agronomic traits, such as stress resilience, a rapid life cycle, and high seed oil content, and displays significant genetic variation among genotypes, thereby exhibiting substantial potential for future breeding improvements (Vollmann et al. 2005; Enjalbert et al. 2013; Rodríguez-Rodríguez et al. 2013). Yet, despite the recognized importance of polyploidization in shaping gene regulation, the impact of these processes on tasiRNA biogenesis and function remains entirely unexplored. Furthermore, miR173 has been previously reported in *C. sativa*, whereas the corresponding downstream *TASI/2*-like loci remain uncharacterized (Poudel et al. 2015). Collectively, these agronomic and genomic features make *C. sativa* a suitable system to investigate the evolutionary conservation and functional divergence of *TASI/2*-homologous loci in polyploid species, particularly in the context of *HTT*-mediated thermotolerance, with implication for both fundamental plant biology and allohexaploid crop enhancement strategies.

The aim of this study was to identify the conservation of *TASI/2*-homologous loci and to infer their functionalities in *C. sativa* through comparison with the well-characterized *TASI/2* genes of *A. thaliana*. Therefore, we systematically identified *TASI/2*-like loci (*TASIL/2L*) across twelve *C. sativa* cultivars and examined their genomic configurations. We also confirmed phasing patterns on *TASIL/2L* loci by analyzing publicly available sRNA-seq generated from *C. sativa* cv. Ames1043, and examined predicted target specificities of tasiR255-like sequences toward *HTT* orthologs. Our findings provide a fundamental framework for comparative studies aimed at understanding how sRNA-mediated *TASI/2* regulatory networks have diverged across a broad spectrum of plant lineages, from allohexaploid *C. sativa* to other allopolyploid systems.

Materials and Methods

Genome Sequence of *C. sativa* Cultivars and *A. thaliana*

Chromosome-level genome assemblies for 12 hexaploidy *C. sativa* cultivars (Acsn-226, Ames1043, Borowska, CAM70, CAM116, CN113611, Giessen4, Joelle, Licalla, Lindo, Prytzh, and Svalof) and their corresponding gene annotation files were obtained from the public genome repository Phytozome (<https://phytozome-next.jgi.doe.gov/>, v1.1) (Goodstein et al. 2012). Sequences of *A. thaliana*'s *TASI/2* genes, *HTT* and *PPR* transcripts, together with the amino acid sequences of the encoded *HTT* and *PPR* proteins were

obtained from TAIR (<https://www.arabidopsis.org/>) (Rhee et al. 2003). Mature miR173 sequences were retrieved from miRBase (<https://www.mirbase.org/>) (Kozomara et al. 2019).

Definition and Identification of *TAS1L/2L* Loci

Based on the characteristics of the *TAS1/2* genes in *A. thaliana*, we established criteria to identify *TAS1L/2L*s in *C. sativa* cultivars (Fig. 1). First, the mature miR173 sequence and the *TAS1c* 3'D6(–) tasiRNA sequence from *A. thaliana* were used as queries to scan all chromosomes using seqkit (v2.8.0) (Shen et al. 2024), allowing up to five mismatches for each query sequence. Here, *TAS1c* 3'D6(–) denotes a *TAS1c*-derived tasiRNA located at the sixth 21-nt phased position on the antisense strand downstream of the miR173-guided cleavage site in *A. thaliana*. The permissive mismatch allowance was selected as an initial screening cutoff based on experimental evidence that some plant small RNA targets can retain sRNA-target interactions including up to five mismatches (Schwab et al. 2005). The

subsequent filtering criteria were applied to reduce potential false-positive while retaining the well-characterized features of *A. thaliana* *TAS1/2* loci. Second, loci were retained as *TAS1L/2L* candidates in accordance with three requirements: (i) the two binding sites were located on the same strand, (ii) their arrangement followed a 5'–miR173–*TAS1c* 3'D6(–)–3' orientation, and (iii) the distance between these sites did not exceed 1,000 bp, consistent with the observations for *A. thaliana* *TAS1/2*. Third, the candidates were further classified as *TAS1L* or *TAS2L* according to the presence of *TAS1*- or *TAS2*-derived tasiRNA-like sequences. Specifically, *TAS1*-derived tasiR255-like sequences potentially targeting *HTT* orthologs defined *TAS1L*, while *TAS2*-derived tasiRNAs, including *TAS2* 3'D6(–), *TAS2* 3'D9(–), *TAS2* 3'D11(–), and *TAS2* 3'D12(–)-like sequences, which are experimentally validated to target *PPR* transcripts in *A. thaliana* (Howell et al. 2007), defined *TAS2L*. These *TAS1L/2L*s formed the basis for all subsequent analyses. The representative miR173-, *TAS1c* 3'D6(–), and *TAS2*-derived tasiRNA sequences used in this study are listed in Supplementary Table S1.

Chromosomal Arrangement of *TAS1L/2L*s Across *C. sativa* Cultivars

Next, we examined the chromosomal location of *TAS1L* and *TAS2L* across 12 *C. sativa* cultivars (Fig. 2). For each cultivar, identified *TAS1L/2L*s were assigned to chromosomes 04, 05, and 06, and ordered according to their genomic coordinates. Detailed strand and genomic coordinate information for all *TAS1L/2L*s, including the positions of the miR173- and *TAS1c* 3'D6(–)-binding sites in each cultivar, is provided in Supplementary Table S2. The lengths of each identified *TAS1L/2L* and the distances between adjacent loci from the last nucleotide of the *TAS1c* 3'D6(–)-binding site to the first nucleotide of the miR173-binding site on the same chromosome were computed. Within each chromosome, the *TAS1L/2L*s were indexed as A–E according to their order along the chromosome number, whereas the tandem structure carrying a miR173-disrupted *TAS2L* was exceptionally labeled with the suffix -F to distinguish its structural oddness. Chromosomes 04, 05, and 06 were further assigned to *C. neglecta* ($n=6$), *C. hispida* ($n=7$), and unidentified subgenomes ($n=7$) (Mandakova and Lysak 2022).

Genomic Configuration and Sequence Conservation of miR173 and tasiRNAs in *TAS1L/2L*s

To examine the genomic configuration of the *TAS1L/2L*s, we screened each *TAS1L/2L* in all *C. sativa* cultivars for tasiRNAs derived from *A. thaliana* *TAS1/2*, which have been experimentally validated to target *HTT* and *PPR*

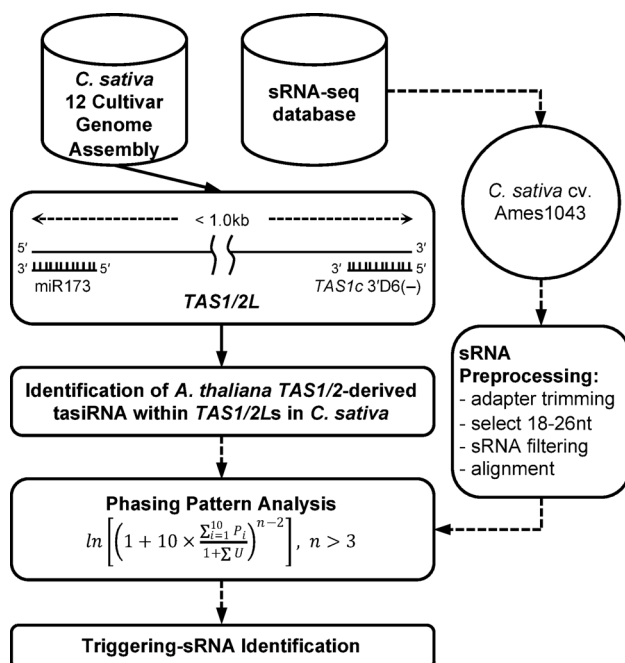


Fig. 1 Pipeline for identifying trans-acting small interfering RNA (tasiRNA)-generating gene-like (*TAS1L/2L*) loci in twelve *Camelina sativa* cultivars. Genome assemblies of twelve *Camelina sativa* cultivars were combined with *Arabidopsis thaliana* *TAS1/2* reference sequences to identify *TAS1L/2L*. Candidate loci were defined as genomic regions containing a miR173-binding site and *TAS1c* 3'D6(–)-binding site on the same strand and within 1.0 kb. Candidate *TAS1L/2L*s were then classified as *TAS1L* or *TAS2L* based on the presence of *TAS1*- or *TAS2*-derived tasiRNA-like sequences, respectively. For the cultivar Ames1043, publicly available sRNA-seq reads were processed and mapped to the identified *TAS1L/2L*s, and phasing pattern analysis was performed to evaluate miR173-registered 21-nt phased siRNA production

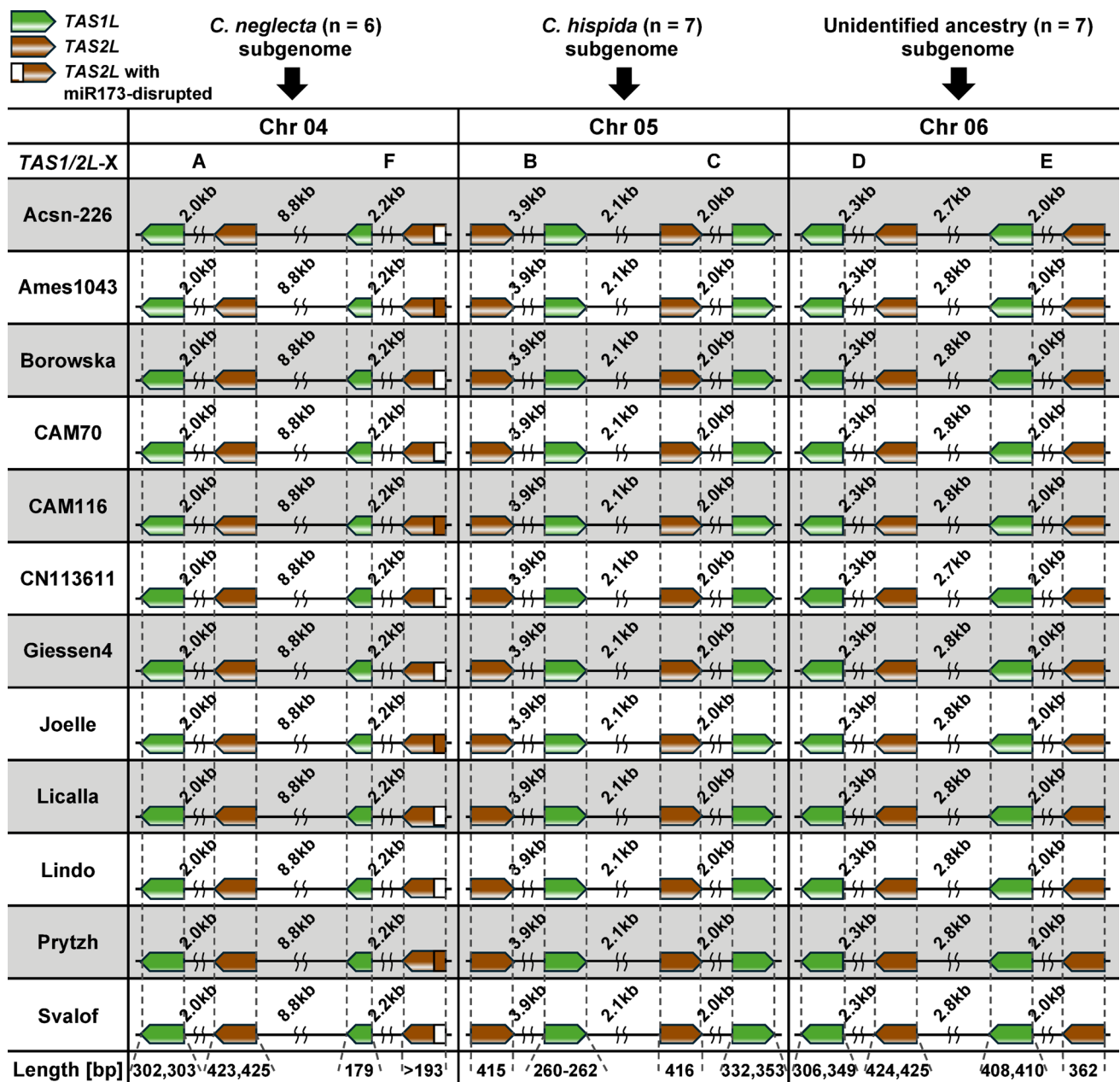


Fig. 2 Schematic diagram of *TAS1/2*-like loci (*TAS1L/2Ls*) preserving tandem structure across twelve *C. sativa* cultivars. *TAS1L* and *TAS2L* loci identified in twelve *C. sativa* cultivars are shown in green and brown, respectively, on (Chr) 04, 05, and 06, corresponding to the *C. neglecta* (n=6), *C. hispida* (n=7), and unidentified ancestry (n=7)

transcripts (*tasiR255*, *TAS2* 3'D6(-), 3'D9(-), 3'D11(-), and 3'D12(-) (Howell et al. 2007) (Fig. 3). For each locus, we examined whether these *tasiRNA*-like sequences were positioned in the expected 21 nt register downstream of the *miR173* cleavage site and then schematically plotted them using TBtools (v2.136) (Chen et al. 2020). Specifically, as the structures of *TAS1L/2Ls* are nearly identical but only with the absence of *sRNAs* in some cultivars, Ames1043 and CAM70 were plotted as the representative cultivar's

subgenomes, respectively. The identified *TAS1L/2Ls* exhibited the conserved *TAS2-TAS1* tandem structure containing *TAS2L-F* with a disrupted *miR173*-binding site located 2.2 kb downstream of *TAS1L-F* on Chr 04. The presence of *A. thaliana* *TAS2*-derived *tasiRNA*-like sequences suggests a minimum length of *TAS2L-F* of 193 bp

configuration to remove unnecessary redundancies. They were plotted in an aligned *TAS2-TAS1* tandem structure arrangement (Fig. 3A). We next used MUSCLE (Edgar 2004) to compare the conservation of key regulatory *sRNAs* within *TAS1L/2Ls* (i.e., the *miR173*-binding site, *tasiR255*-like sequences, and *TAS1c* 3'D6(-)-like sequences) across all cultivars with those of *A. thaliana*. Specifically, as the sequences in *C. sativa* were almost identical across all cultivars, only Ames1043 and CAM70 cultivars were used as

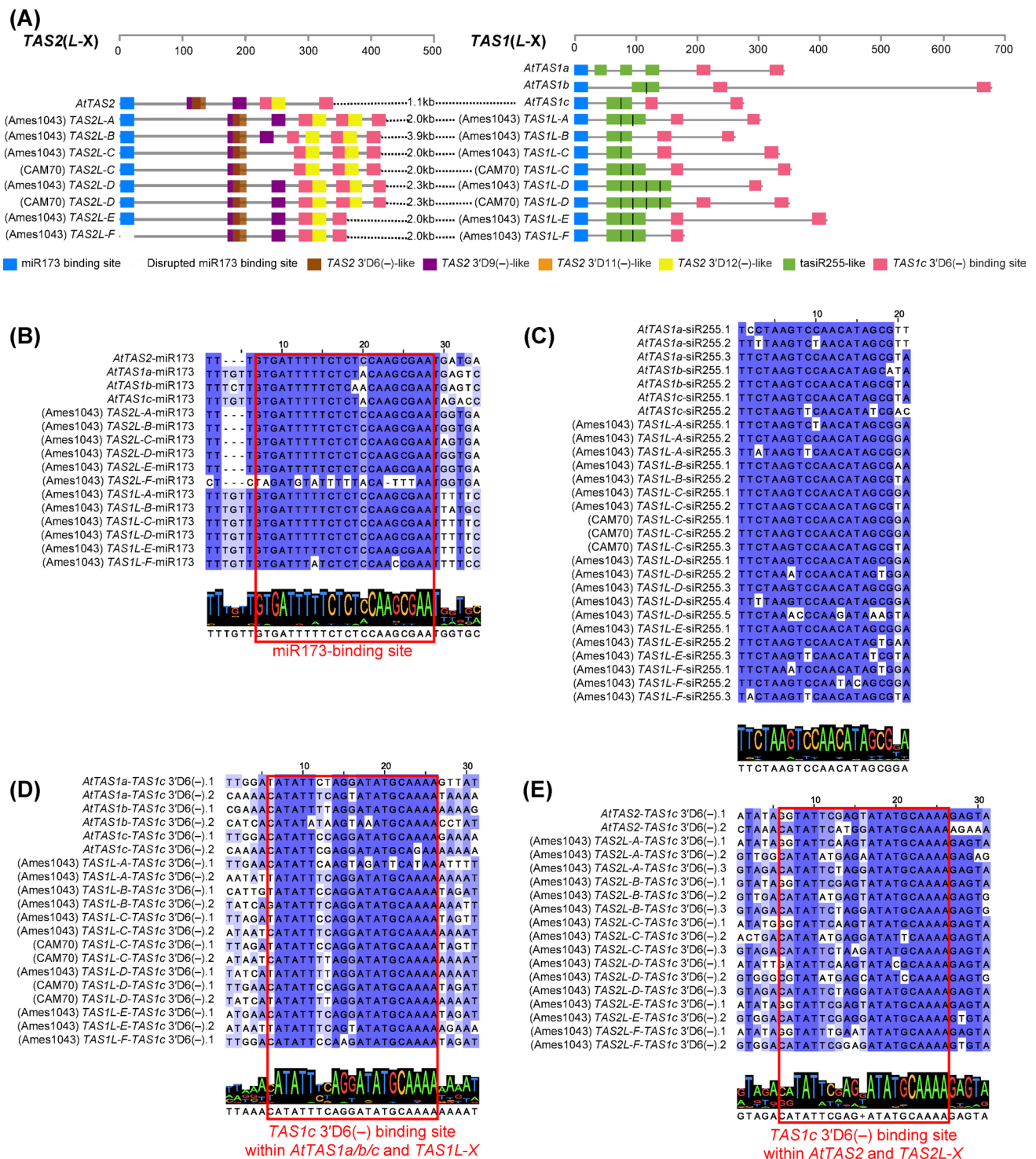


Fig. 3 Genomic structures of *TAS2L-TAS1L* tandems and the conservation of essential small RNAs in twelve *C. sativa* cultivars and *A. thaliana*. **A** Schematic representation of *TAS2-TAS1* tandem loci in *A. thaliana* and representative *TAS2L-TAS1L* tandems from the *C. sativa* cultivars Ames1043 and CAM70, showing duplication of *TAS1c* 3'D6(-)-like sequences in *TAS2L* and multiple copies of tasiR255-like sequences (up to five) in *TAS1L-D*. **B-E** Multiple sequence alignments

of **(B)** miR173-binding sites, **(C)** tasiR255-like sequences, **(D)** *TAS1c* 3'D6(-)-binding sites in *AtTAS1a/b/c* and *TAS1L-X*, and **(E)** *TAS1c* 3'D6(-)-binding sites in *AtTAS2* and *TAS2L-X* from 12 *C. sativa* cultivars. This demonstrates strong conservation of these essential sRNA-related regions in the miR173-*TAS1/2* regulatory pathway between *A. thaliana* and *C. sativa* *TAS* loci

representatives in the plots to avoid unnecessary redundancies, such as the genomic configuration of *C. sativa*. The representative cultivars represent all the different characteristics of all 12 cultivars (Fig. 3B–E).

Phasing Pattern Analysis of *TAS1L/2Ls*

15 publicly available sRNA-seq datasets from wild-type, *rdr2* and *nrpel* plants for *C. sativa* cultivar Ames1043 were obtained from the NCBI BioProject database (PRJNA986946) (Dew-Budd et al. 2024). Adapter sequences were removed using Trimmomatic (v0.39) and Cutadapt (v4.9) (Martin 2011; Bolger et al. 2014). To eliminate structural non-coding RNAs, the trimmed reads were aligned to sequences of plant rRNA, tRNA, and snRNA (housekeeping RNAs) retrieved from RNA central (<https://rnacentral.org/>) (v24) using Bowtie (v1.3.1) with the parameters (-v 1 -m 0 -a) (Langmead et al. 2009; The RNAcentral Consortium 2019). Unaligned reads were subsequently extracted and realigned to the *C. sativa*'s genome sequences using Bowtie (-v 0 -a -m 2000) to ensure comprehensive mapping.

The phasing patterns were analyzed using a modified version of the algorithm introduced by De Paoli et al. (2009), which is based on the method originally described by Howell et al. (2007), accounting for the depth and potential background noise (Fig. 4). Phasing scores were calculated to assess the periodicity of sRNA processing using the following equation:

$$\text{Phase Score} = \ln \left[\left(1 + 10 \times \frac{\sum_{i=1}^{10} P_i}{1 + \sum U} \right)^{n-2} \right], \quad n > 3$$

where n is the number of phase cycle registers containing at least one sRNA read within a 10-cycle window (phase cycle length was set to 21 nt), P is the total number of reads for all sRNAs with 5' ends in a given phase within a 10-cycle window, and U is the total number of reads for all sRNAs with the 5' ends out of the given phase within the window. Pairs of sRNAs from the sense and antisense strands, offset by 2 nt with the overhang at the 3' ends, were combined.

Moreover, the downstream region of the miR173-guided cleavage site at each *TAS1L/2L* was partitioned into 21 bins corresponding to the 21 nt phasing cycle. The nucleotide immediately after the cleavage site was assigned to bin 1, the next to bin 2, and so on to bin 21, with positions further mapped according to $((k-1) \bmod 21) + 1$, where k is the distance from the cleavage site. Reads mapped to the antisense strand were also considered by explicitly accounting for their 2 nt overhang.

Investigation of the tasiRNA-Targeted Proteins HTT and PPR

We first focused on identifying orthologs of the *HTT* genes encoded in *A. thaliana* (*AtHTT1–6*), which are known targets of tasiR255 (Li et al. 2014). Using these AtHTT amino acid sequences as queries in a Phytozome BLASTP search (v2.11.0+) (Altschul et al. 1997), we screened 12 *C. sativa* cultivars and retained protein hits with an E-value $\leq 1\text{E-}70$. The selected sequences were aligned with MUSCLE, and alignment refinement was performed with trimAl (v1.5.0) (Capella-Gutierrez et al. 2009) to remove poorly aligned regions. Next, we constructed a maximum likelihood phylogenetic tree with RAxML-NG (v1.2.2) (Kozlov et al. 2019), using 1000 bootstrap replicates to cluster the proteins into distinct branches. Given the almost identical sequences within the same cluster in the case of our datasets, a representative sequence was chosen based on its frequency for each branch. These representative sequences were then jointly aligned with the AtHTT1–6 sequences using MUSCLE and trimmed using trimAl, followed by phylogenetic tree construction using RAxML-NG with parameters --model VT+G4 --bs-trees 1000. To predict the targeting specificity, we applied psRNATarget (Dai et al. 2018) with specific penalties for G:U pairs (0.5) and other mismatches (1.0), and the predicted interactions were visualized as a heatmap (Fig. 5A). A complete list of clustered *C. sativa* HTT ortholog proteins and their predicted tasiR255-like target sites (including target coordinates and sequences) is provided in Supplementary Table S3.

For PPR proteins, we employed a similar strategy. Experimentally validated phasing PPR sequences (Howell et al. 2007) were used as queries in a Phytozome BLASTP search across the 12 *C. sativa* cultivars. After filtering for hits with an E-value of less than $1\text{E-}70$, we analyzed the resulting sequences using the Phase Score equation to determine their phasing patterns and the corresponding triggering tasiRNAs. This comprehensive pipeline enabled us to systematically assess both HTT and PPR protein families, thereby elucidating the conservation and functional roles of tasiRNA-targeted genes in *C. sativa*.

Results and Discussion

Identification of *TAS1L/2Ls* in the Genomes of 12 *C. sativa* Cultivars

We used miR173 and *TAS1c* 3'D6(–) sequences of *A. thaliana* as queries to screen the genomes of twelve *C. sativa* cultivars. This resulted in a list of hundreds of putative miR173-binding sites and thousands of *TAS1c* 3'D6(–)-binding sites from each genome of *C. sativa*'s cultivar. To narrow down the candidate

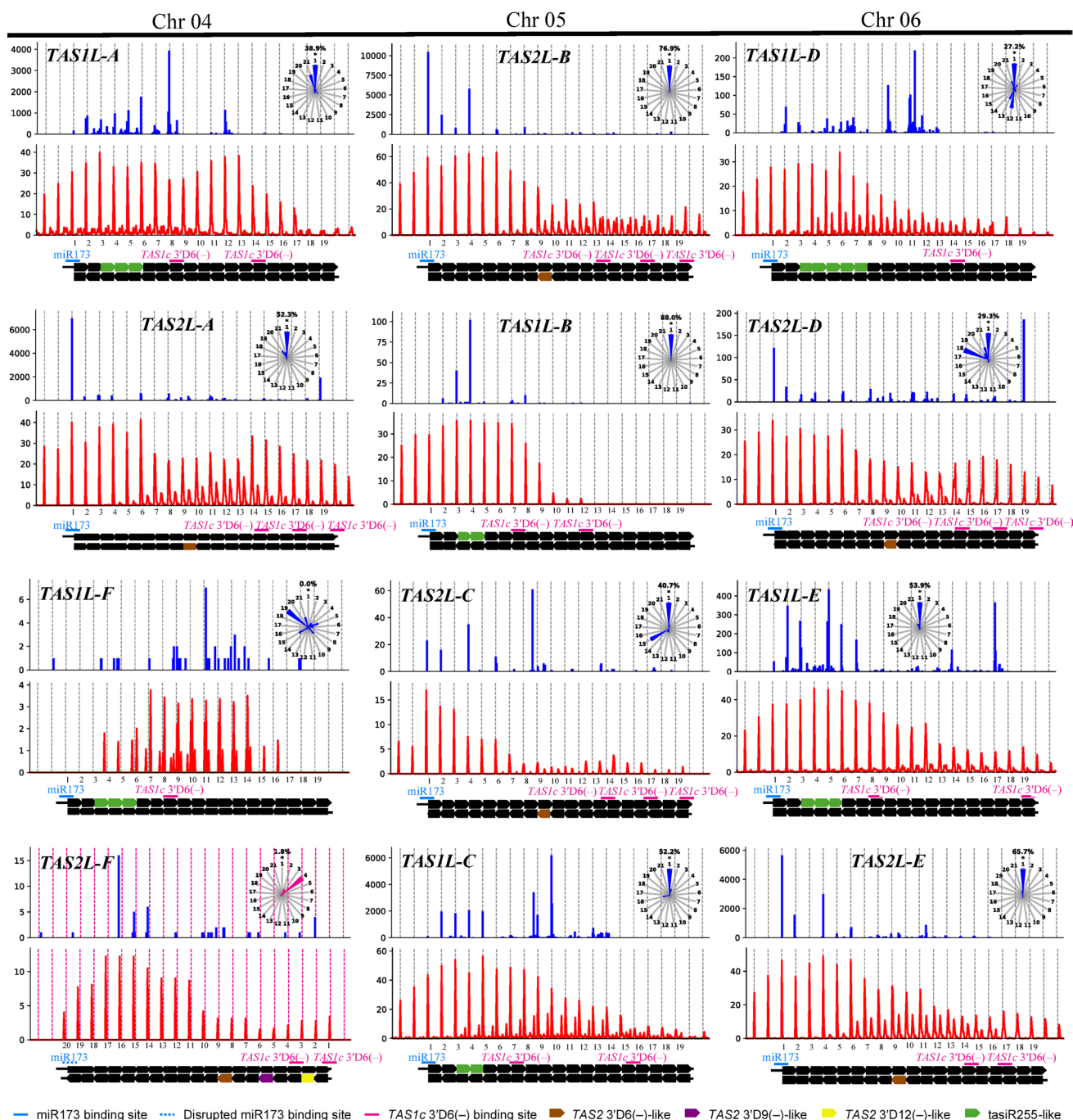


Fig. 4 Distribution of 21-nucleotide small RNAs in *TASIL/2Ls* with conserved *TAS2-TAS1* tandem structure in *C. sativa* cv. Ames1043. The top graphs (blue) display the total number of small RNA reads with a radial graph showing the percentages of the reads corresponding to each of the 21 registers, based on sRNA-seq data from *C. sativa* cv. Ames1043. Register-1 is defined by the 5'-end formed by miR173-guided cleavage site. The bottom graphs (red) display the phasing signals. The y-axis represents the phasing scores calculated based on the results of 21 nt small RNAs using the phasing score function. In both graphs, the 21 nt phased positions initiated by miR173-guided cleavage are indicated by gray dotted lines. The schematics below the

phasing plots represent the 21 nt phased-register arrangement of each *TASIL/2L* and the positions of tasiRNA-like sequences within these registers. For *TAS2L-F*, because the miR173-binding site is disrupted, the 21 nt phased positions and the schematic registers were assigned based on the terminal *TAS1c* 3'D6(-)-binding site, as indicated by pink dotted line. In these schematics, thin solid lines indicate the miR173- and *TAS1c* 3'D6(-)-binding sites, whereas the blue dotted lines indicate disrupted miR173-binding site. Black arrows indicate consecutive 21 nt phased registers, whereas colored arrows indicate tasiRNA-like sequences positioned within the registers, as shown in the labels below

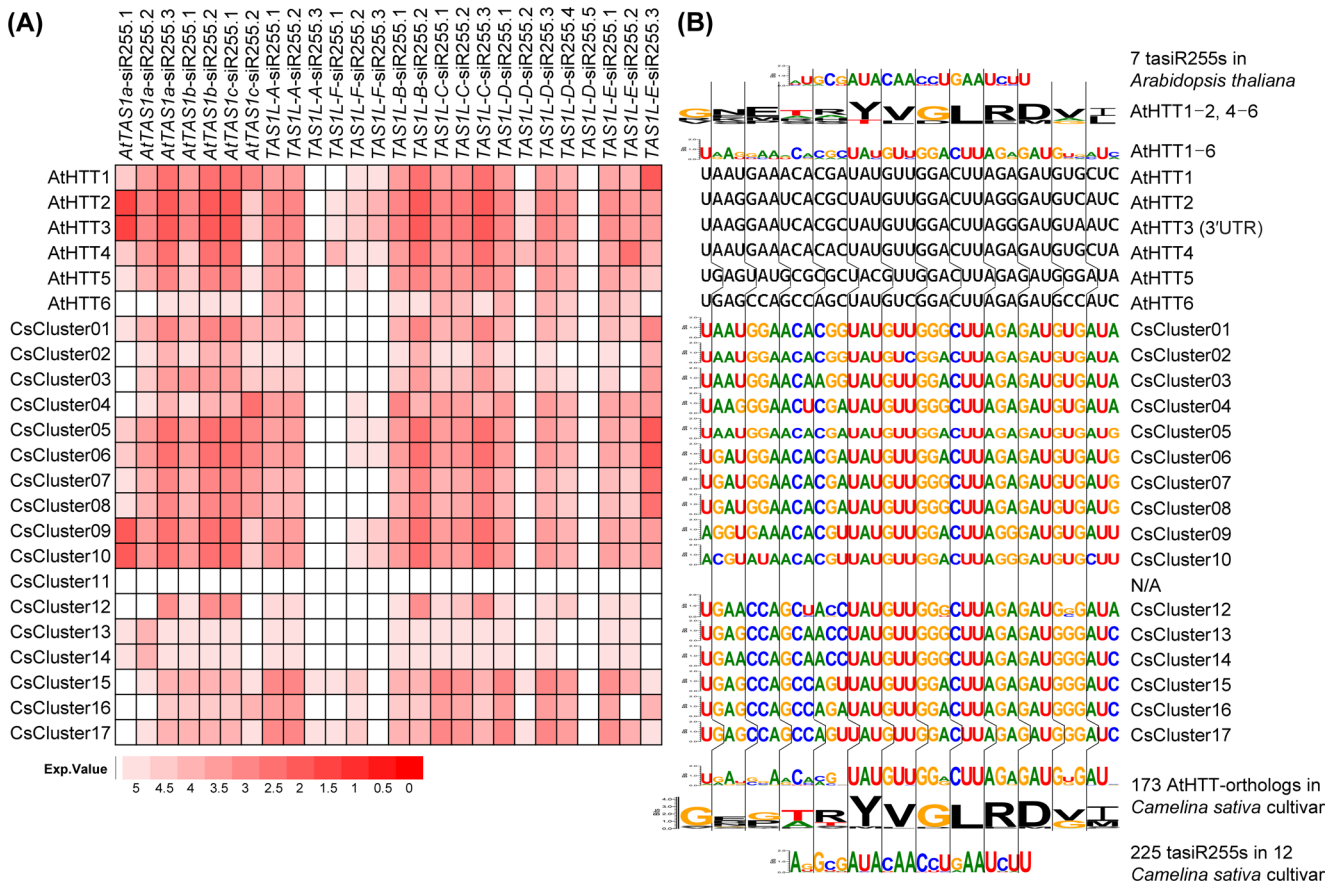


Fig. 5 Predicted targeting of *HEAT-INDUCED TAS1 TARGET* (*HTT*) orthologs by *TASIL*-derived tasiR255-like small interfering RNAs in *A. thaliana* and *C. sativa*. **A** Heatmap of psRNA target expectation scores showing predicted interactions between seven *AtTAS1*-derived tasiR255s and 225 *TASIL*-derived tasiR255-like siRNAs from 12 *C. sativa* cultivars with *AtHTT1*–6 and 17 *C. sativa* HTT clusters. Lower expectation scores indicate higher predicted targeting efficiency. Most

TAS1- or *TAS2*-homologous genomic regions from searched loci, several criteria referred from features of *TAS1/2* genes of *A. thaliana* were applied (see Materials and Methods). We then classified these candidate loci as *TASIL* or *TAS2L* based on the similarity of tasiRNA sequences with those on *A. thaliana*'s *TAS1* or *TAS2* (listed in Supplementary Table S1). As a result, we found 5 *TAS2L* (*TAS2L-A–E*) loci and 6 *TASIL* (*TASIL-A–F*) loci satisfying all criteria from each *C. sativa* cultivar's genome (Fig. 2). We also found one location that contains the *TAS1c* 3'D6(–)-binding site and the *A. thaliana* *TAS2*-encoding tasiRNA sequences but does not have the miR173-binding site satisfying the search criterion (mismatch ≤ 5-nt). Hence, we manually searched the upstream of this putative *TAS2*-like locus and found a region that has a disrupted miR173-binding site (Fig. 2, *TAS2L* in "F" column). After further investigation of sequence similarity for its nearby region, we concluded that this exceptional case corresponds to the *TAS2*-like and included to a list of *TASIL/2L* for our downstream analysis.

HTT clusters showed low expectation scores for multiple tasiR255 or tasiR255-like siRNAs, whereas CsCluster11 lacked any recognizable tasiR255 target site. *TASIL-F*-derived tasiR255-like siRNAs and terminally duplicated copies in *TASIL-A* and *TASIL-D* showed weak or no predicted targeting. **B** Summary of tasiR255-like target site distribution across HTT clusters in *A. thaliana* and *C. sativa*, highlighting the conserved YVGLRD-encoding target-regions of *HTT* transcripts

Conservation of *TAS1L/2L*s in Twelve *C. sativa* Cultivars at the Chromosomal Level

Next, we explored genomic arrangement of *TASIL* and *TAS2L* loci identified in *C. sativa* cultivars as a chromosomal perspective. The six *TASIL* and *TAS2L* loci were distributed with two copies each on three homeologous chromosomes of *C. sativa*, Chr04, Chr05 and Chr06 (Fig. 2), which correspond to the *C. neglecta* ($n=6$), *C. hispida* ($n=7$), and unidentified ancestry subgenomes, respectively (Mandakova and Lysak 2022). Considering *C. sativa* is assumed to be an evolutionary descendant of those three *Camelina* plants, the observed uniformed genomic distribution of *TASIL* and *TAS2L* copies across these homeologous chromosomes suggests that both *TASIL* and *TAS2L* are highly supposed to be conserved in ancestry plants of *C. sativa*.

Overall, the lengths of these *TASIL/2L* loci ranged 260–425 bp except for *TASIL-F*, which was considerably shorter

(179 bp) (Fig. 3A and Supplementary Table S2). Notably, the relative location between *TAS1L* and *TAS2L* exhibited a tandem arrangement in the order of *TAS2L–TAS1L* across all *C. sativa* cultivars, which is similar to the *TAS2–TAS1c* arrangement in the genome of *A. thaliana* (Yoshikawa et al. 2005). Within each *TAS2L–TAS1L* arrangement, the order and transcriptional orientation *TAS1L/2L* were identical among all twelve cultivars and were also same with the *A. thaliana*'s *TAS2–TAS1c*. Additionally, the intergenic distances within each *TAS2L–TAS1L* tandem and the intervals between two tandems on individual chromosomes showed remarkable conservation across all cultivars. These features indicate that the tandem organization of *TAS1L/2Ls* has been strongly conserved at the subspecies level, and at the family level as well given that the *TAS2–TAS1* tandem structure was observed from both *A. thaliana* and *C. sativa*. To this end, the triplication of *TAS2L–TAS1L* tandem structures is likely due to the allohexaploidization occurred during the emergence of *C. sativa*.

Conservation of the Genomic Configuration of *TAS1L/2Ls* at the Sequence Level

Given the conserved chromosomal arrangement, we next investigated the genomic configuration of *TAS1L/2Ls* at the sequence level. Multiple sequence alignments revealed that, aside from minor 1–2 nucleotide mismatches, each *TAS1L* and *TAS2L* sequence was highly conserved across all *C. sativa* cultivars. We therefore plotted two representative cultivars, Ames1043 and CAM70, which showed clear differences in these loci (Fig. 3A). These differences were attributed to two specific variations in certain loci: *TAS1L-C* and *TAS1L-D* exhibited a 21 nt and 43 nt difference, respectively, across all *C. sativa* cultivars. The variation in *TAS1L-C* resulted from the lack of a 21 nt tasiR255-like sequence (crucial for regulating *HTT* transcripts in *A. thaliana*) in three cultivars, whereas this sequence was present in the nine cultivars. Similarly, the variation in *TAS1L-D* stemmed from the absence of a *TAS1c* 3'D6(–)-like sequence (crucial for regulating PPR proteins) in five cultivars and present in seven. These observations suggest that, although the overall *TAS1/2* system is structurally conserved across *C. sativa* cultivars, these loci may show a potential capacity for secondary phasing and possible cultivar-specific variation in regulatory mechanisms.

To further characterize the *TAS1L/2L* system, we first examined the structures of *TAS2Ls* (Fig. 3A). As in *A. thaliana* *TAS2*, *C. sativa* *TAS2Ls* contained sites corresponding to *TAS2* 3'D6(–), *TAS2* 3'D9(–), *TAS2* 3'D11(–), and *TAS2* 3'D12(–) in the same order. Notably, in *C. sativa*, the *TAS1c* 3'D6(–)-binding sites with *TAS2* 3'D12(–)-like sequences were duplicated, which may contribute to the production of

multiple targeting siRNAs through secondary phasing by *TAS1c* 3'D6(–)-like sequences, which is consistent with the mechanism of secondary phasing in *A. thaliana* (Rajeswaran et al. 2012). We next examined *TAS1L*, which is defined by the presence of a tasiR255-like sequence (Fig. 3A). Remarkably, the tasiR255-like sequence was massively expanded, forming up to five continuous copies in *TAS1L-D*. Furthermore, the *TAS1c* 3'D6(–)-like sequence, which is known to trigger secondary phasing in *A. thaliana*, was consistently present in duplicate at the 3' side of most *TAS1L* loci, suggesting a potential capacity for secondary phasing at these loci and possible cultivar-specific regulatory mechanisms.

We further confirmed that the miR173-binding site, which initiates phasing, is highly conserved in *TAS1L/2Ls* across all 12 cultivars, except in the case of *TAS2L-F*, where the site is disrupted (Fig. 3B). The miR173-binding site was highly similar to the experimentally validated sequence in *A. thaliana*, indicating that it is likely to trigger effectively as an initiation site for tasiRNA production in *C. sativa*. The tasiR255-like sequences, aside from a single nucleotide variation at position 20, were also highly similar to *A. thaliana* tasiR255, suggesting that they can potentially target *HTT* ortholog transcripts (Fig. 3C). Inspection of the *TAS1c* 3'D6(–)-binding sites in *TAS1Ls* further showed that all cultivars harbor *TAS1c* 3'D6(–)-like sequences that could be cleaved and thereby induce secondary phasing (Fig. 3D). In *TAS2Ls*, compared to *TAS1Ls*, the 3' side was well conserved across *TAS1L/2Ls*, whereas the 5' side in *TAS2Ls* was less conserved than that in *TAS1Ls* (Fig. 3E). Because the seed region remains highly similar, these sites still represent potential regions for secondary phasing, and variation in the surrounding sequences may contribute to diversification of tasiRNA-mediated regulation.

Phasing pattern analysis

With our above observations, we could know that all twelve *C. sativa* cultivars possess conserved *TAS1L/2Ls* arranged in tandem structure on three chromosomes, mirroring some structural characteristics observed in *A. thaliana*. It was therefore natural to ask whether these loci are functionally active, like their *Arabidopsis* counterparts. To confirm this question, we analyzed the phasing patterns of sRNAs generated from the miR173-guided cleavage site on *TAS1L/2Ls* using sRNA-seq data and calculated phasing scores as described in the Methods section (Fig. 4), presenting the full set of loci to provide an overview of the overall phasing patterns. Because publicly available sRNA-seq data were available only for the cultivar Ames1043, the phasing analysis presented here provides functional support only for *TAS1L/2Ls* in Ames1043. Thus, the conservation observed across the remaining cultivars suggests structural

conservation rather than direct functional validation. Further sRNA-seq analyses of additional *C. sativa* cultivars would help clarify whether the phasing patterns observed in Ames1043 are conserved across other cultivars.

In Ames1043, sRNAs were detected exclusively in the downstream region of the miR173 cleavage site, indicating a unidirectional processing mechanism (Howell et al. 2007) (Fig. 4). Furthermore, the dominant phasing pattern precisely aligned with the miR173 cleavage register, with phased sRNAs predominantly clustered toward the miR173-binding region, suggesting that miR173 is the initiator of tasiRNA production. Notably, as the phasing cycle progressed, occasional 1–2 nt mismatches were observed (e.g., in *TAS1L-A*), a phenomenon reminiscent of the phase drift previously reported in *A. thaliana* (Howell et al. 2007). These resembled features suggest that, as in *A. thaliana*, the phasing patterns are generated, at least in part, by DICER-LIKE 4 and possibly by alternative dicer-like enzymes.

Intriguingly, the *TAS1L-F* locus, which is roughly half the length of other *TAS1L/2Ls*, exhibited markedly low levels of sRNA expression and an aberrant phasing pattern. Likewise, the *TAS2L-F* locus also accumulated only very few sRNA reads. Because *TAS2L-F* carries a disrupted miR173-binding site, these weak and irregular phasing signatures are most plausibly explained by a failure to initiate miR173-guided cleavage, providing additional support that miR173 acts as the primary trigger for tasiRNA biogenesis in *C. sativa*. Consistent with this interpretation, the *TAS1L/2L-F* tandem appears largely functionally attenuated compared with the other *TAS1L/2Ls* (Fig. 4). We further detected weak phasing patterns that did not align with the miR173-initiated register in *TAS2L-A*, *TAS2L-B*, *TAS1L-D*, *TAS1L-C*, and *TAS2L-D*. These minor phasing registers corresponded to secondary phasing initiated at *TAS1c* 3'D6(–)-like sequences (Supplementary Figure S1), where phase drift was likewise observed. Together, these results show that *TAS1L/2Ls* in Ames1043 are processed under the influence of miR173 to produce 21 nt phased siRNAs, although the loci differed in their phasing pattern, including phase drift, the presence of minor secondary registers, and the strength of phasing at the *TAS1L/2L-F* tandem.

In *A. thaliana*, *TAS2*-derived tasiRNAs are known to predominantly target *PPR* transcripts, prompting us to inspect whether *TAS2L*-derived phased siRNAs similarly engage with *PPR* ortholog transcripts (Supplementary Figure S2). Among the 95 *PPR* orthologs in Ames1043, 25 exhibited phasing patterns, and four represented regions potentially targeted by *TAS2* 3'D6(–), *TAS2* 3'D9(–), *TAS2* 3'D11(–), *TAS2* 3'D12(–), and *TAS1a* 3'D9(–)-like sequences. Therefore, the observed phasing patterns of *PPR* orthologs suggest that *TAS2L*-derived tasiRNAs may be associated with potential regulation of *PPR* transcripts in *C. sativa* cv.

Ames1043, consistent with the *TAS2*–*PPR* relationship in *A. thaliana* (Howell et al. 2007). Having examined the potential *PPR* regulation associated with *TAS2L*-derived tasiRNAs, we next focused on *HTT* orthologs because *TAS1L*-derived tasiR255-like sequences were notably expanded in *TAS1Ls* (Fig. 3A), and tasiR255 is known to regulate *HTT* transcripts in *A. thaliana*.

Analysis of *HTT* Regulated by *TAS1L*-Derived tasiR255-like Sequences

The expanded replication of tasiR255-like sequences in *C. sativa*, together with evidence of the phasing patterns observed from Ames1043, suggests potential functional significance in gene regulation. We therefore analyzed the predicted targeting efficiency of *TAS1L*-derived tasiR255-like sequences on *HTT* ortholog protein regulation (Fig. 5A). This analysis was further motivated by the established function of tasiR255 in *A. thaliana* (Li et al. 2014), i.e., modulation of *HTT* expression to confer thermotolerance. To assess the predicted targeting efficiency of *TAS1L*-derived tasiR255-like sequences, we analyzed their complementarity to *HTT* clusters across twelve *C. sativa* cultivars and *A. thaliana* (Supplementary Table S3). Comparative analysis between the two genomes suggested that most tasiR255-like sequences exhibited cross-genus targeting efficiency, supporting a conserved regulatory mechanism. However, a notable exception was observed in CsCluster11, which lacked any recognizable tasiR255 target sites.

Further examination of *TAS1L*-derived tasiR255-like sequences suggested differences in predicted targeting capacity among different loci (Fig. 5A). Specifically, tasiRNAs generated from the *TAS1L-F* locus displayed significantly decreased targeting efficiency toward *HTT* transcripts compared to those from other *TAS1Ls*. Additionally, the third tasiR255-like sequence from *TAS1L-A* and the fifth from *TAS1L-D* failed to effectively target most *HTT* sequences. Notably, all these ineffective tasiRNAs were located at the terminal replicated regions of their corresponding *TAS1Ls*, suggesting the potential influence of duplication patterns on tasiRNA efficiency and the broader evolutionary constraints shaping their regulatory roles.

Additionally, analysis of 173 *HTT* genes across the twelve *C. sativa* cultivars revealed that the targeted region, particularly the amino acid motif YVGLRD, is remarkably well conserved compared to other regions of the *HTT* proteins (Fig. 5B). This pattern of conservation is similarly observed in *A. thaliana*, in which the targeted sequences in each *HTT* cluster are nearly identical at the amino acid level. However, when clusters are compared, the YVGLRD region consistently stands out for its exceptional conservation. Collectively, these findings are consistent with a

conserved tasiR255–*HTT* regulatory relationship and suggest that sequence conservation may help maintain this predicted interaction across species. We also note that, because this analysis was based on the sequence complementarity, additional transcriptomic analyses and experimental validation will be required.

Conclusions

In this study, we extended the miR173–*TAS1/2* regulatory circuit beyond *A. thaliana* by systematically defining and characterizing *TAS1L/2Ls* in twelve cultivars of the allohexaploid oilseed crop *C. sativa*. Using the definition with colocalized miR173 and *TAS1c* 3'D6(–)-like trigger sites together with *TAS1*- and *TAS2*-derived tasiRNA-like sequences, we uncovered a conserved genomic organization in which six *TAS1L/2Ls* are arranged as three *TAS2L*–*TAS1L* tandems corresponding to the three subgenomes across all cultivars. Locus order, transcriptional orientation, and intergenic spacing were highly conserved. Within this otherwise highly conserved organization, notably, tasiR255- and *TAS1c* 3'D6(–)-like sequences, which play a crucial role in the regulation of *HTT* and *PPR* in *A. thaliana*, showed variation. This suggests potential cultivar-specific variation in the components that shape tasiRNA production and targeting during *Camelina* polyploid evolution.

Small RNA phasing analysis in Ames1043 further provided functional support for the processing of *TAS1L/2Ls* into miR173-registered 21 nt phased siRNAs, broadly consistent with the miR173–*TAS1/2* characteristics in *A. thaliana*. The phasing patterns also included occasional phase drift and secondary phasing initiated by *TAS1c* 3'D6(–)-like sequences, as previously observed for *TAS1/2* loci in *A. thaliana*. Target prediction further suggested that *TAS1L*-derived tasiR255-like sequences may broadly target *HTT* transcripts across *C. sativa* and *A. thaliana*, and the strong conservation of the YVGLRD motif in *HTT* proteins is consistent with a constrained interaction. The absence of recognizable tasiR255 target sites in specific *HTT* clusters and the decreased targeting capacity of some terminally duplicated tasiR255-like copies suggest ongoing diversification within the tasiR255–*HTT* network.

Future sRNA-seq analyses of additional *C. sativa* cultivars will further clarify whether the phasing patterns observed in Ames1043 are conserved across cultivars. More broadly, our *TAS1L/2L* identification strategy, combined with phasing analysis and target prediction, provides a practical framework for dissecting tasiRNA pathways in polyploid genomes and may be extended to other tasiRNA systems across plant lineages.

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Data Availability This study did not generate new datasets. All data analyzed were from publicly available sources as described in the Materials and Methods.

Declarations

Conflict of interest The authors declare no conflict of interest.

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