Genome-wide identification and characterization of miRNAs responsive to *Verticillium longisporum* infection in *Brassica napus* by deep sequencing

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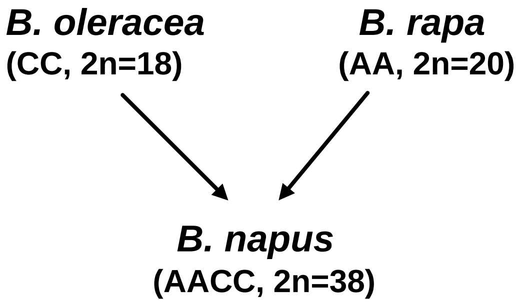
Brassica napus

- An important source for edible vegetable oil and biodiesel worldwide.

- A newly formed amphidiploid species, belongs to Brassicaeae

- Originating from hybridizations between *B. rapa* and *B. oleracea.*

- The diseases of rapeseed crop:
    - *Verticillium* wilt
    - Blackleg
    - Sclerotinia stem rot



B. oleracea
(CC, 2n=18)

B. rapa
(AA, 2n=20)

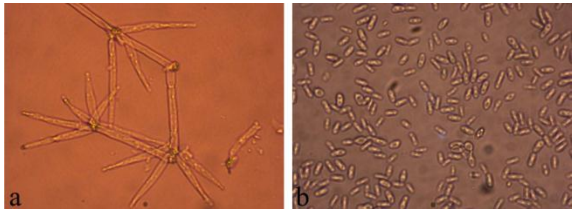
B. napus
(AACC, 2n=38)

Verticillium wilt in *B. napus*

- Soil-borne plant fungus
- A narrow host range limited to *Brassicaceae*
- A long infection process
- Stunted growth, leaf chlorosis, early flowering and senescence
- No effective resistance in *Brassicaceae*

The rapeseed -*V. longisporum* interaction

a unique system for investigation:



- ➢ Plant-pathogen interaction
- ➢ Molecular and physiological reprogramming, post-transcriptional gene silencing (PTGS)
- ➢ Gene regulation: **miRNAs**

miRNA: biogenesis and function

- A class of short, near-ubiquitous and endogenous RNAs of ~21 nucleotides

- Functional component: RISC (miRNA + AGO1 protein)

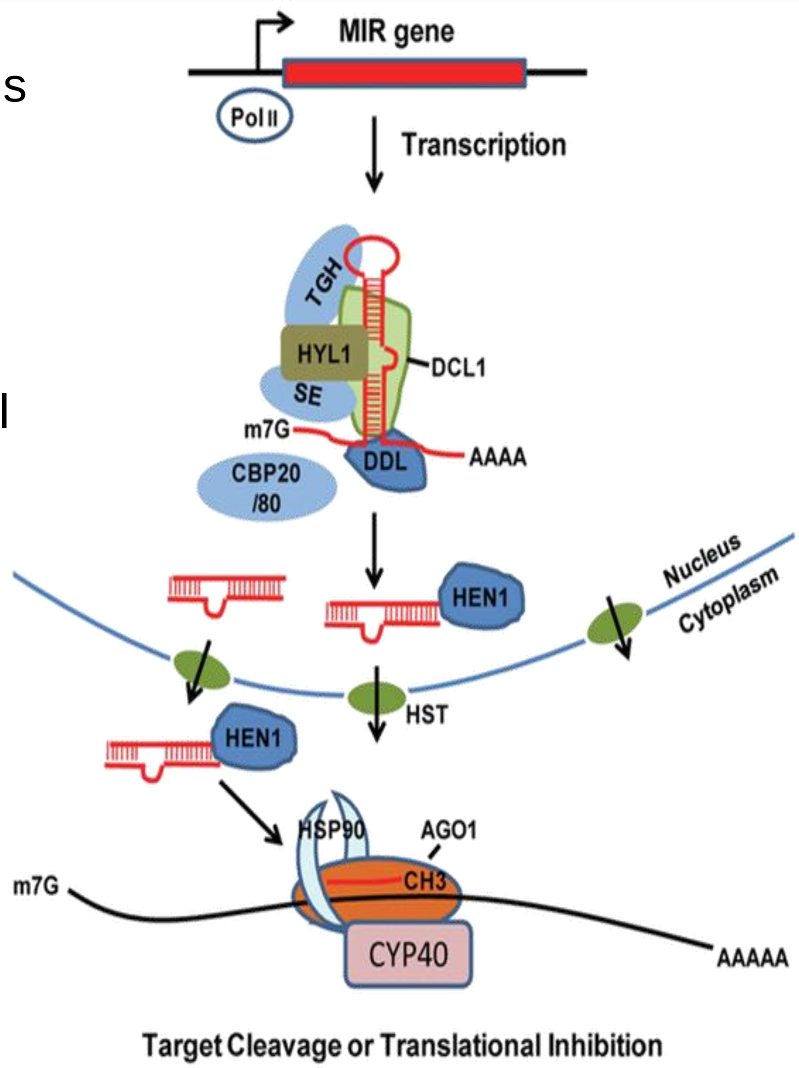
- Action model: mRNA degradation or translational inhibition

- Roles:

  Post-transcriptiponal gene regulation

  - plant growth and development
  - abiotic stress
  - biotic stress

(Khraiwesh *et al*. 2011)



Working and Aims:

Hypothesis

Plant miRNAs are essential regulators controlling the *B. napus-V. longisporum* interaction by direct or indirect interference with plant defence responses.

Aims:

- Genome-wide identification of miRNAs in *Brassica napus* based on *B. rapa* (AA) and *B. oleracea* (CC) genome sequences.

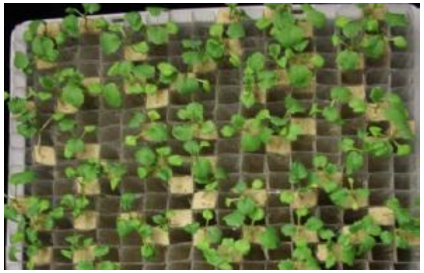
- Molecular evolution of miRNAs in *Brassica napus.*

- Identification and characterization of miRNAs responsive to *V. longisporum* infection.

- Possible action model of miRNAs responsive to *V. longisporum* infection.

Infection experiments

Express 617



VL+ **VL-**

Working steps

UR
6-dpi control roots

IR
6-dpi infected roots

Small RNA sequencing

Data filtering and analysis

- miRNA identification
- Expression validation
- Secondary structure prediction
- Target prediction and validation

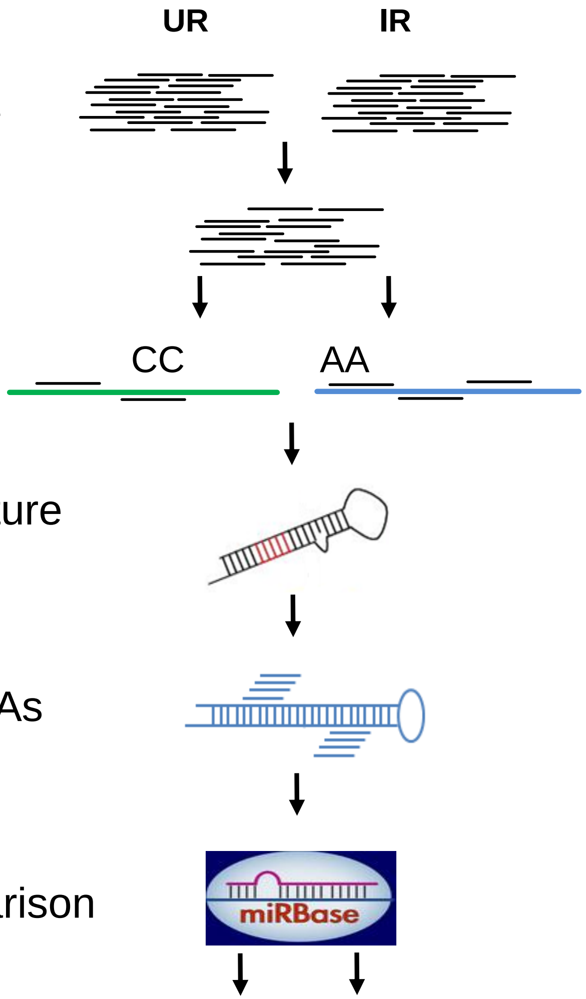
Microsynthenic analysis

Evolutionary origin and relationship demonstration

Differential expression analysis

Functional characterization

miRNA identification by deep-sequencing



Sequencing data

Data filtering

mapping

Secondary structure prediction

Candidate miRNAs

Database comparison

Conserved miRNAs

B. napus novel miRNAs

UR : un-infected roots
IR: infected roots

Conserved miRNAs:
homological to plant miRNAs recorded in the miRBase

***B. napus* novel miRNAs:**
only found in *B. napus*

miRNAs of *B. napus*

www.mirbase.org (Release 20)

B. napus: 92
B. rapa: 43
B. oleracea: 7
Arabidopsis: 337
Oryza sativa: 713
Homo sapiens: 2,578

Identification of miRNAs from *B. napus* (AACC):

Before 2010:

EST sequences

TC sequences

BAC sequences

A. thaliana sequences

After 2010:

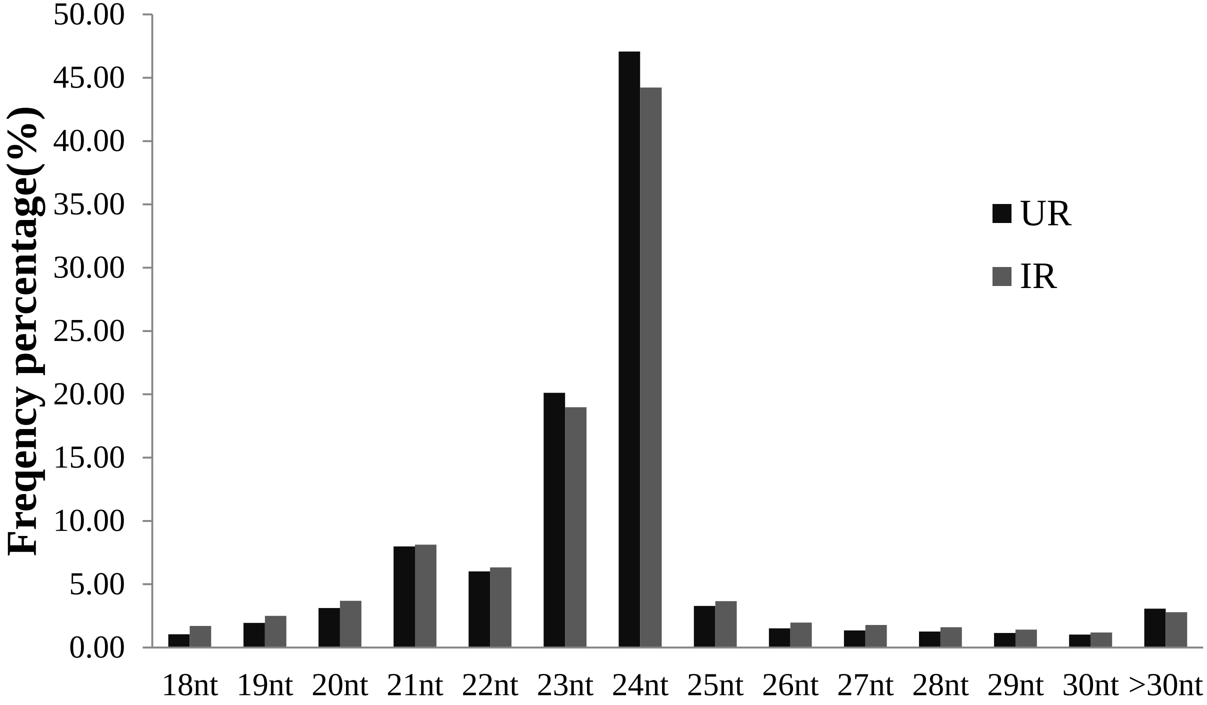
Brassica rapa (AA) genome sequence (2011)

Brassica oleracea (CC) genome sequence (2013)

Small RNAs from two sequenced libraries (UR and IR)

Small RNAs	**UR**		**IR**	
	Unique	**Abundant**	**Unique**	**Abundant**
Total number of reads	**5,085,622**	**16,926,646**	**4,223,911**	**17,157,110**
Mapped to *B. rapa or B. oleracea* genome	**2,335,615** **(45.9%)**	**9,899,731** **(58.5%)**	**1,851,246** **(43.8%)**	**8,354,172** **(48.7%)**
B. rapa	1,192,848 (23.5%)	7,107,303 (42.0%)	955,669 (22.6%)	6,109,185 (35.6%)
B. oleracea	1,820,776 (35.8%)	8,797,610 (52.0%)	1,443,201 (34.2%)	7,369,932 (43.0%)
Mapped to *B. rapa* or *B. oleracea* transcripts	474,831 (9.3%)	1,510,735 (8.9%)	379,536 (9.0%)	1,125,027 (6.6%)
B. rapa	267,038 (5.3%)	855,763 (5.1%)	214,143 (5.1%)	633,906 (3.7%)
B. oleracea	301,118 (5.2%)	1,021,962 (6.0%)	242,075 (5.7%)	760,040 (4.4%)

Size distribution of two sequenced libraries (UR and IR)



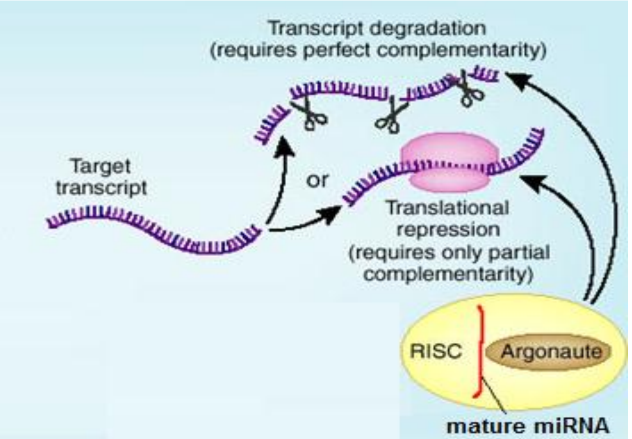
Size distribution of small RNA (unique reads) from two libraries

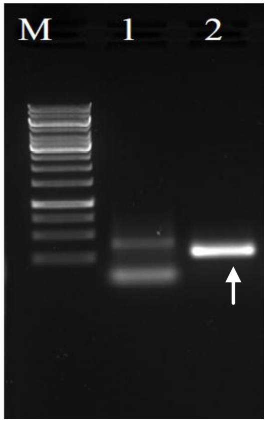
B. napus miRNAs identified from two libraries

Origin	Conserved miRNAs	Novel miRNAs	Total
miRNAs in AA	**194**	**235**	**429**
miRNAs in CC	**166**	**298**	**464**
Total	**360**	**533**	**893**

- 360 conserved and 533 *B. napus* novel or specific miRNAs were identified.
- *B. rapa* (A A) and *B. oleracea* (CC) genomes almost donate an equal number of miRNAs to *B. napus.*

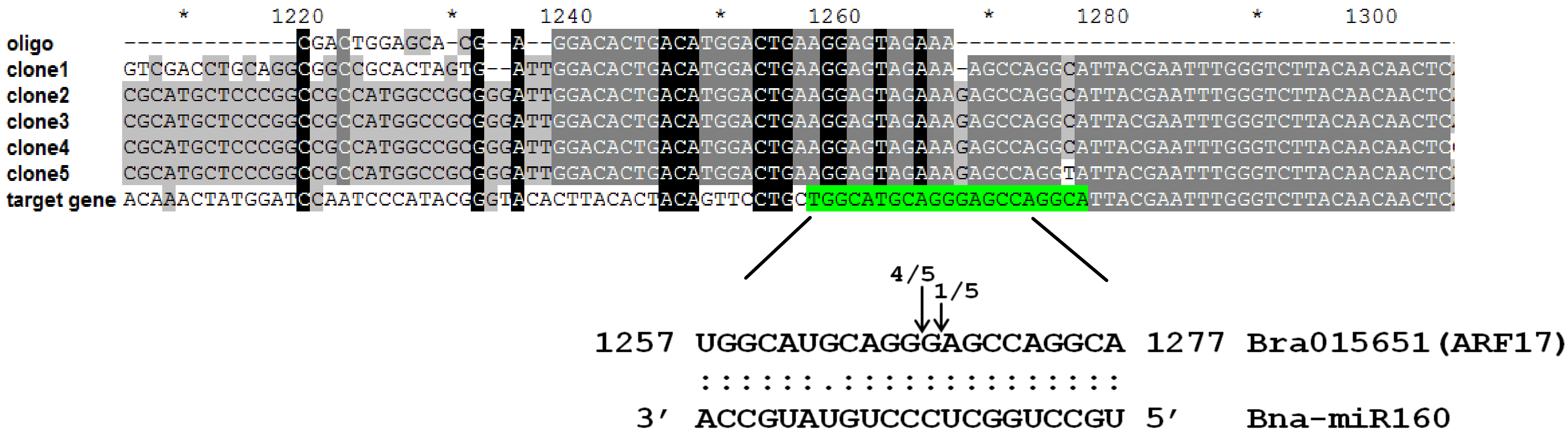
miRNA target validation: 20 miRNA targets have been validated





5’ RACE based identification of miRNA target genes of *B. napus*

M: 1kb ladder
1: touch-down PCR for miR160 target, Bra015651
2: nested PCR miR160 for miR160 target, Bra015651
The arrow-marked nested-PCR products cloned for sequencing



```
                *      1220         *      1240         *      1260         *      1280         *      1300
oligo       -------------CGACTGGAGCA-CG--A--GGACACTGACATGGACTGAAGGAGTAGAAA-------------------------------------
clone1      GTCGACCTGCAGGCGGCCGCACTAGTG--ATTGGACACTGACATGGACTGAAGGAGTAGAAA-AGCCAGGCATTACGAATTTGGGTCTTACAACAACTC
clone2      CGCATGCTCCCGGCCGCCATGGCCGCGGATTGGACACTGACATGGACTGAAGGAGTAGAAAGAGCCAGGCATTACGAATTTGGGTCTTACAACAACTC
clone3      CGCATGCTCCCGGCCGCCATGGCCGCGGATTGGACACTGACATGGACTGAAGGAGTAGAAAGAGCCAGGCATTACGAATTTGGGTCTTACAACAACTC
clone4      CGCATGCTCCCGGCCGCCATGGCCGCGGATTGGACACTGACATGGACTGAAGGAGTAGAAAGAGCCAGGCATTACGAATTTGGGTCTTACAACAACTC
clone5      CGCATGCTCCCGGCCGCCATGGCCGCGGATTGGACACTGACATGGACTGAAGGAGTAGAAAGAGCCAGGTATTACGAATTTGGGTCTTACAACAACTC
target gene ACAAACTATGGATCCAATCCCATACGGGTACACTTACACTACAGTTCCTGCTGGCATGCAGGGAGCCAGGCATTACGAATTTGGGTCTTACAACAACTC
```

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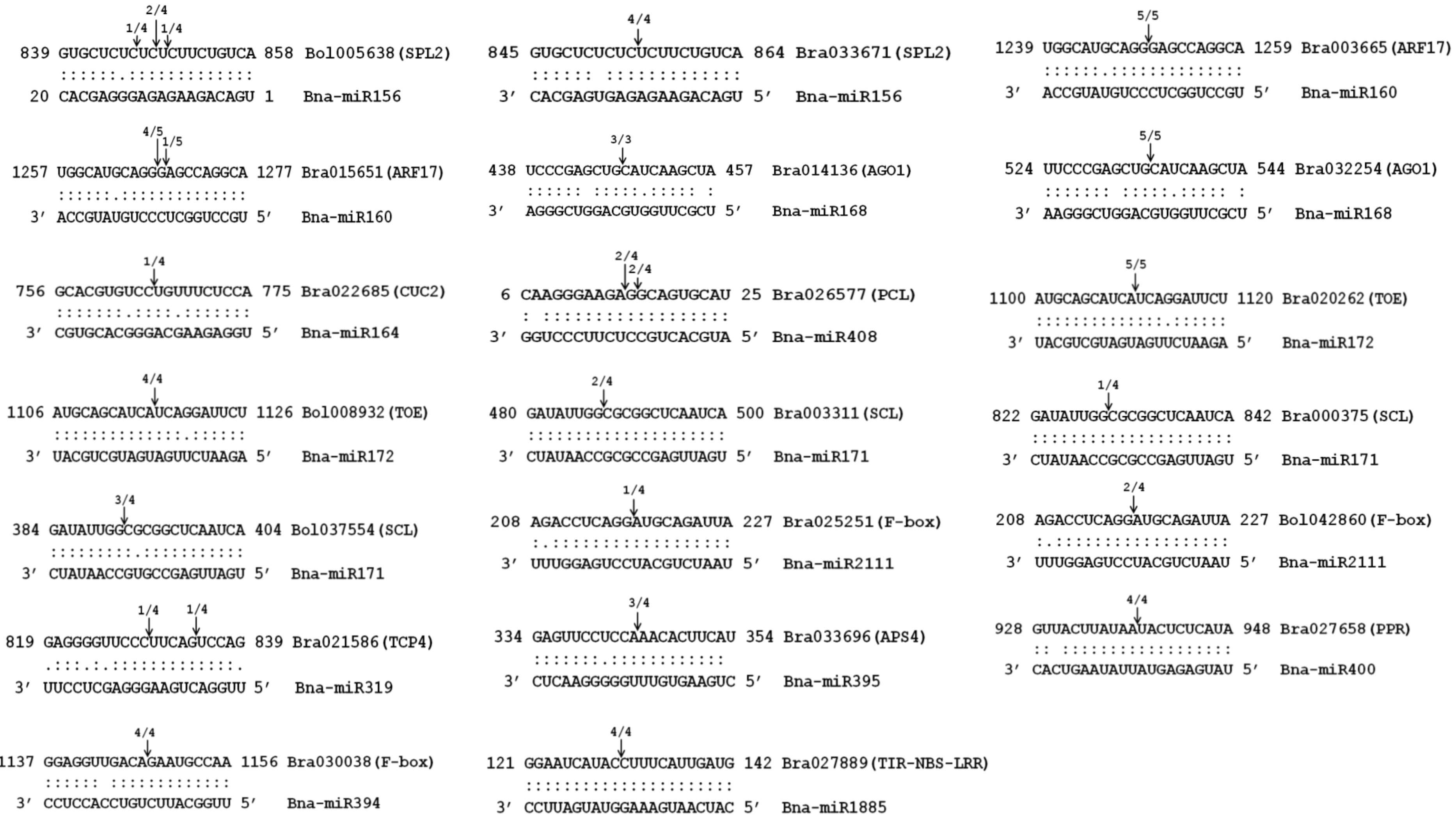
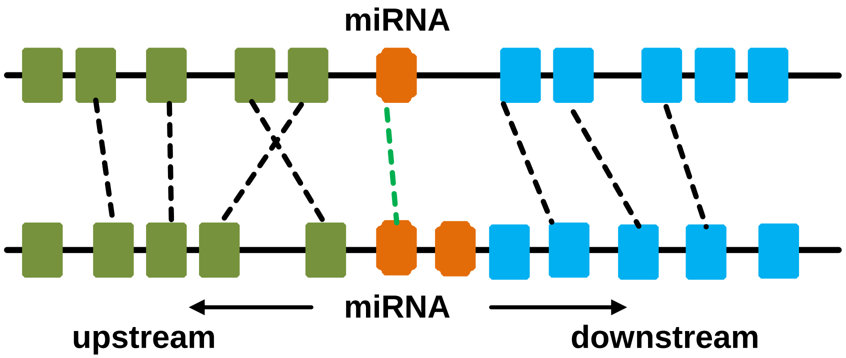
              4/5
               1/5
1257 UGGCAUGCAGGGAGCCAGGCA 1277 Bra015651(ARF17)
     ::::::.::::::::::::::
  3' ACCGUAUGUCCCUCGGUCCGU 5'   Bna-miR160
```

miRNA targets: 20 targets were selected and validated



Synteny analysis

Synteny analysis on the basis of miRNAs and their two flanking protein coding genes revealed genomic synteny of miRNAs between A and C genomes



1. miRNA similarity comparison between AA and CC
2. 10 flanking protein coding gene of miRNA similarity comparison between AA and CC
3. Determine syntenic miRNAs

Table: Synteny of conserved miRNAs identified by this study

Syntenic type	miRNA_AA	miRNA_CC
Both streams	128	
Single stream	9	
None stream	33	
AA /CC specific	61	42

Both loci: both upstream and downstream have protein-coding gene orthology
Single loci: either upstream or downstream has protein-coding gene orthology
None loci: neither upstream nor downstream has protein-coding gene orthology
Specific: AA- or CC- genome specific miRNAs

AA/CC syntenic analysis of *B. napus* miRNA

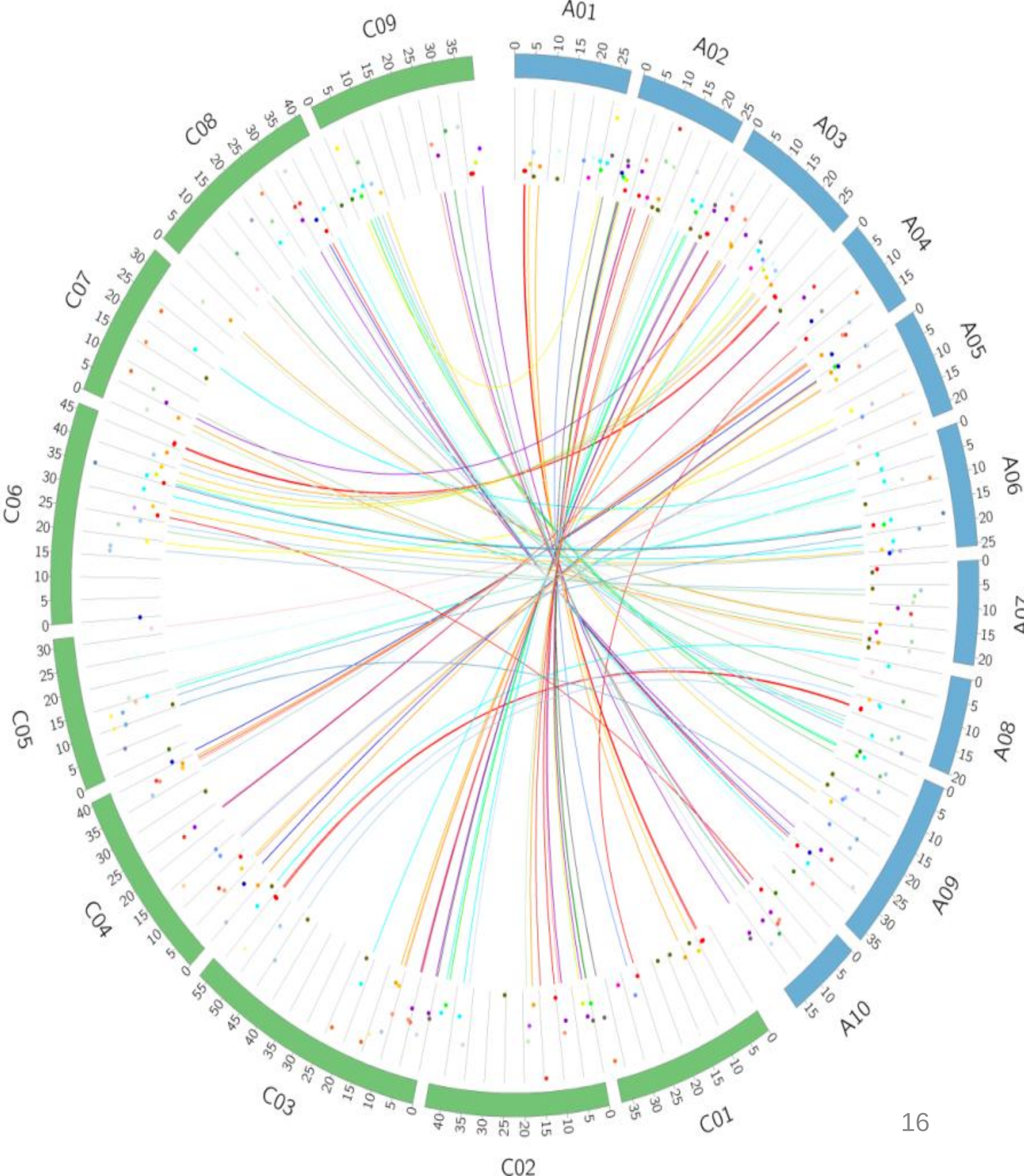
Origin	Type	AA	AA-CC-syntenic pair	CC
Conserved miRNAs	Syntenic miRNAs	137	(137)	137
	Unique miRNAs	61		42
Specific miRNAs	Synteny miRNAs	4	(4)	4
	Unique miRNAs	216		282

- Most of conserved miRNAs from AA and CC genome are paralogous.

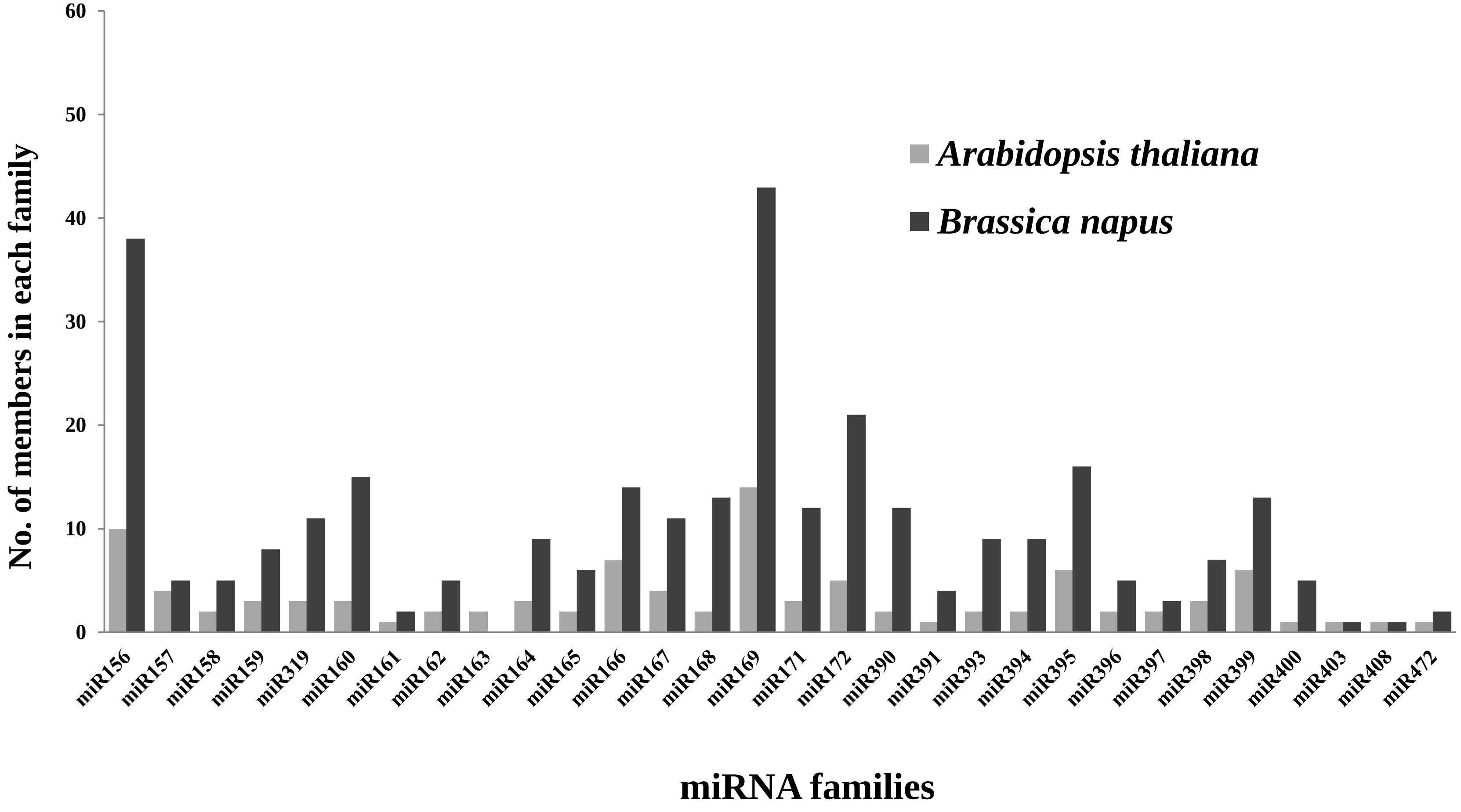
- Most of *B. napus* specific miRNAs are AA-/CC- genome specific.

Syntenic MIRNA loci of conserved miRNAs identified from *B. napus* in *B. rapa* (AA) and *B. oleracea* (CC) genomes.

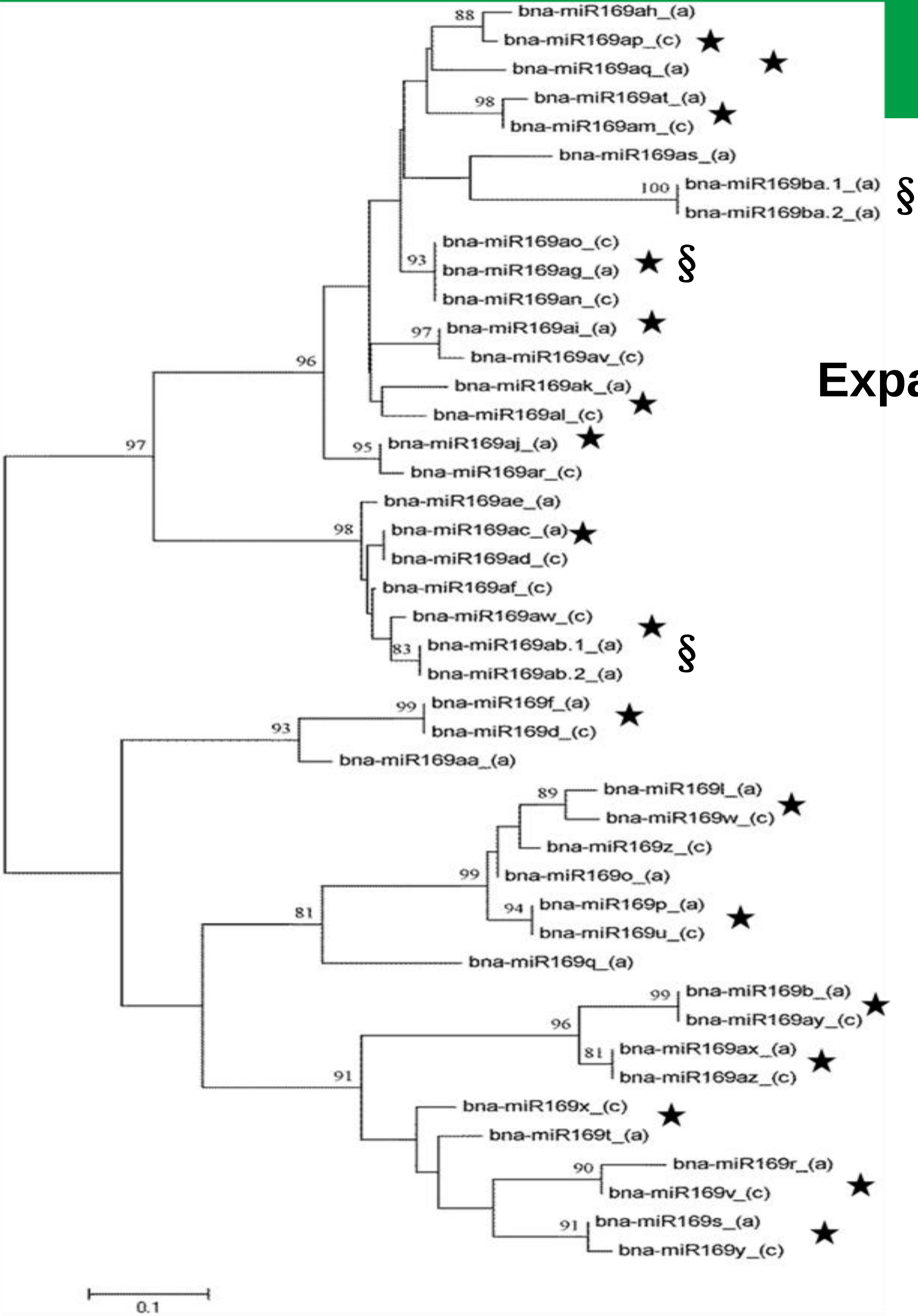
Syntenic miRNA pairs are evenly scattered over the whole chromosomes of *B. napus*



Expansion of miRNA families in *B. napus*

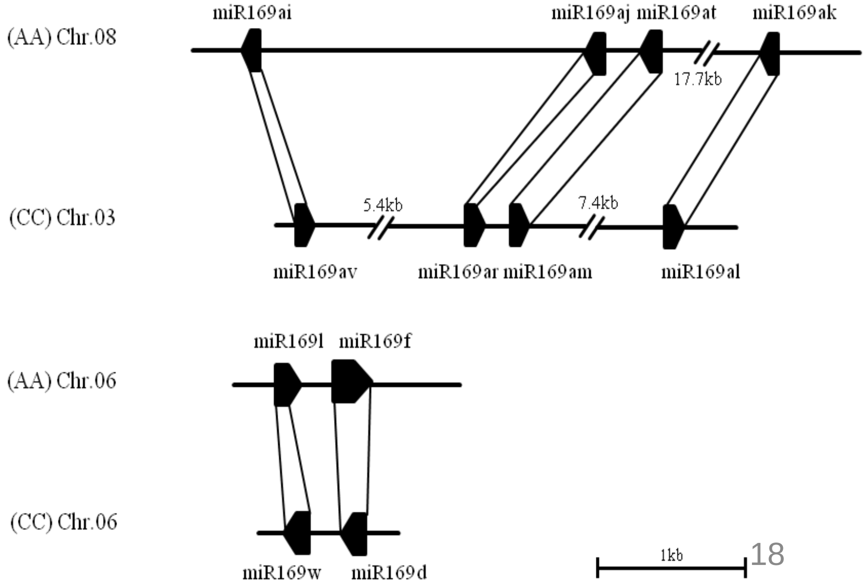


Expansion of miR169 family in *B. napus*

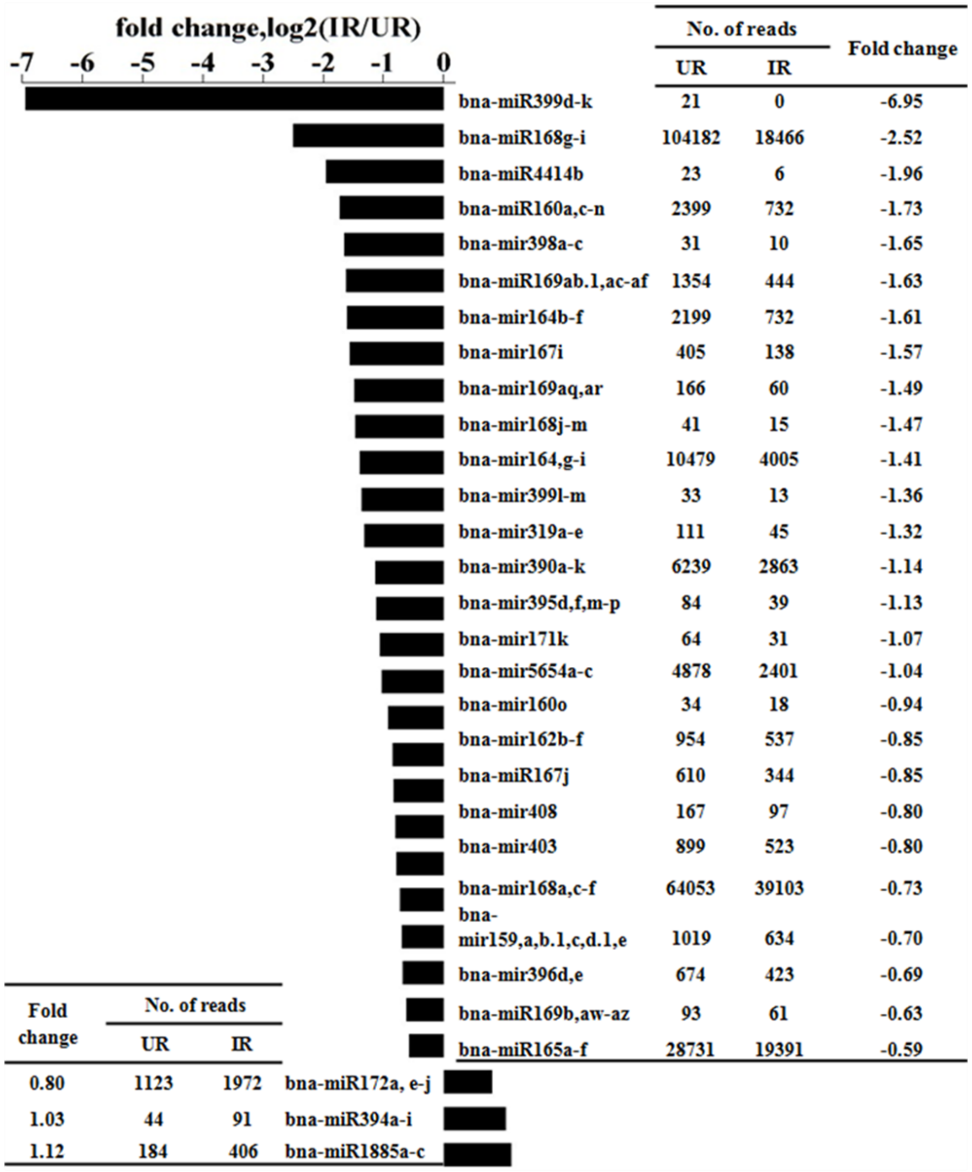


★ Syntenic between AA and CC genome

§ Tandem duplication



Identification of miRNAs responsive to *V. longisporum* infection



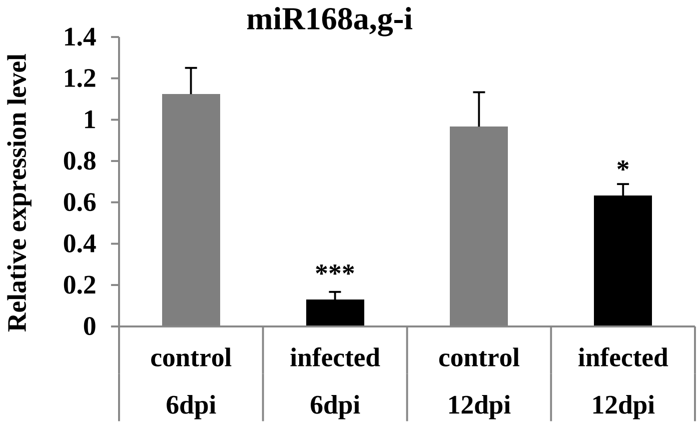
	No. of reads		Fold change
	UR	IR	
bna-miR399d-k	21	0	-6.95
bna-miR168g-i	104182	18466	-2.52
bna-miR4414b	23	6	-1.96
bna-miR160a,c-n	2399	732	-1.73
bna-mir398a-c	31	10	-1.65
bna-miR169ab.1,ac-af	1354	444	-1.63
bna-mir164b-f	2199	732	-1.61
bna-mir167i	405	138	-1.57
bna-mir169aq,ar	166	60	-1.49
bna-mir168j-m	41	15	-1.47
bna-mir164,g-i	10479	4005	-1.41
bna-mir399l-m	33	13	-1.36
bna-mir319a-e	111	45	-1.32
bna-mir390a-k	6239	2863	-1.14
bna-mir395d,f,m-p	84	39	-1.13
bna-mir171k	64	31	-1.07
bna-mir5654a-c	4878	2401	-1.04
bna-mir160o	34	18	-0.94
bna-mir162b-f	954	537	-0.85
bna-miR167j	610	344	-0.85
bna-mir408	167	97	-0.80
bna-mir403	899	523	-0.80
bna-mir168a,c-f	64053	39103	-0.73
bna-mir159,a,b.1,c,d.1,e	1019	634	-0.70
bna-mir396d,e	674	423	-0.69
bna-miR169b,aw-az	93	61	-0.63
bna-miR165a-f	28731	19391	-0.59

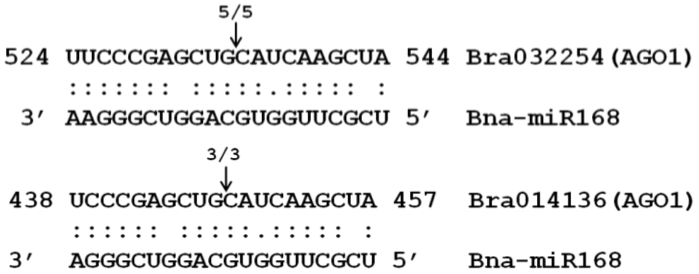
Fold change	No. of reads		
	UR	IR	
0.80	1123	1972	bna-miR172a, e-j
1.03	44	91	bna-miR394a-i
1.12	184	406	bna-miR1885a-c

- 119 conserved miRNAs (20 miRNA families) were down-regulated after *V. longisporum* infection
- 19 conserved miRNAs (3 miRNA families) were up-regulated after *V. longisporum* infection
- The sequencing data can be confirmed by qRT-PCR analysis
- Target genes are dominantly involved in:
    - ➢ Stimuli response
    - ➢ Development
    - ➢ Metabolic processes

A set of miRNAs were responsive to VL infection

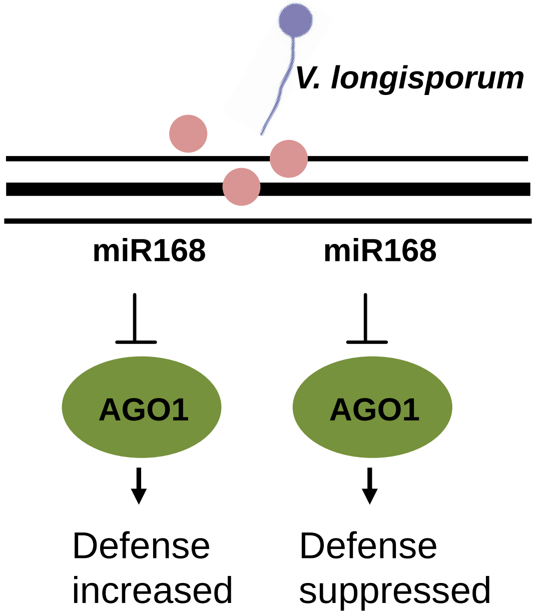
miRNA	**Target**	**Roles**	**Response to *VL***
miR168	AGO1	miRNA synthesis, virus infection…	Down-regulated
miR164	CUC2	Leaf formation…	Down-regulated
miR160	ARF17	Regulate early auxin response gene…	Down-regulated
miR1885	NBS-LRR domain	Plant resistance gene	Up-regulated





```
                5/5
                 ↓
524 UUCCCGAGCUGCAUCAAGCUA 544 Bra032254(AGO1)
    ::::::: :::::.::::: :
 3′ AAGGGCUGGACGUGGUUCGCU 5′  Bna-miR168

               3/3
                ↓
438 UCCCGAGCUGCAUCAAGCUA 457  Bra014136(AGO1)
    :::::: :::::.::::: :
3′  AGGGCUGGACGUGGUUCGCU 5′   Bna-miR168
```



V. longisporum

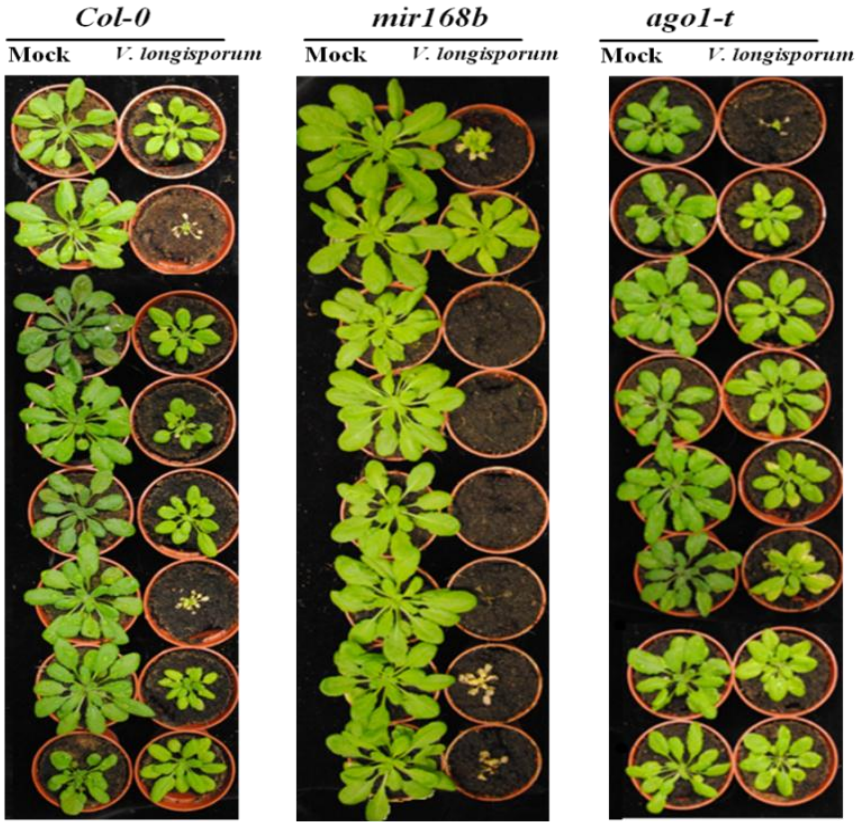
miR168 **miR168**

AGO1 **AGO1**

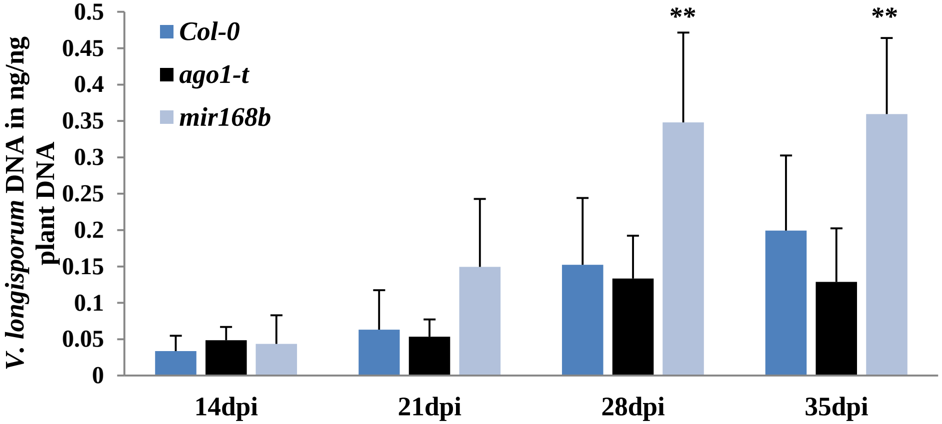
Defense increased

Defense suppressed

miR168- AGO1 interference determines the susceptibility to VL-infection



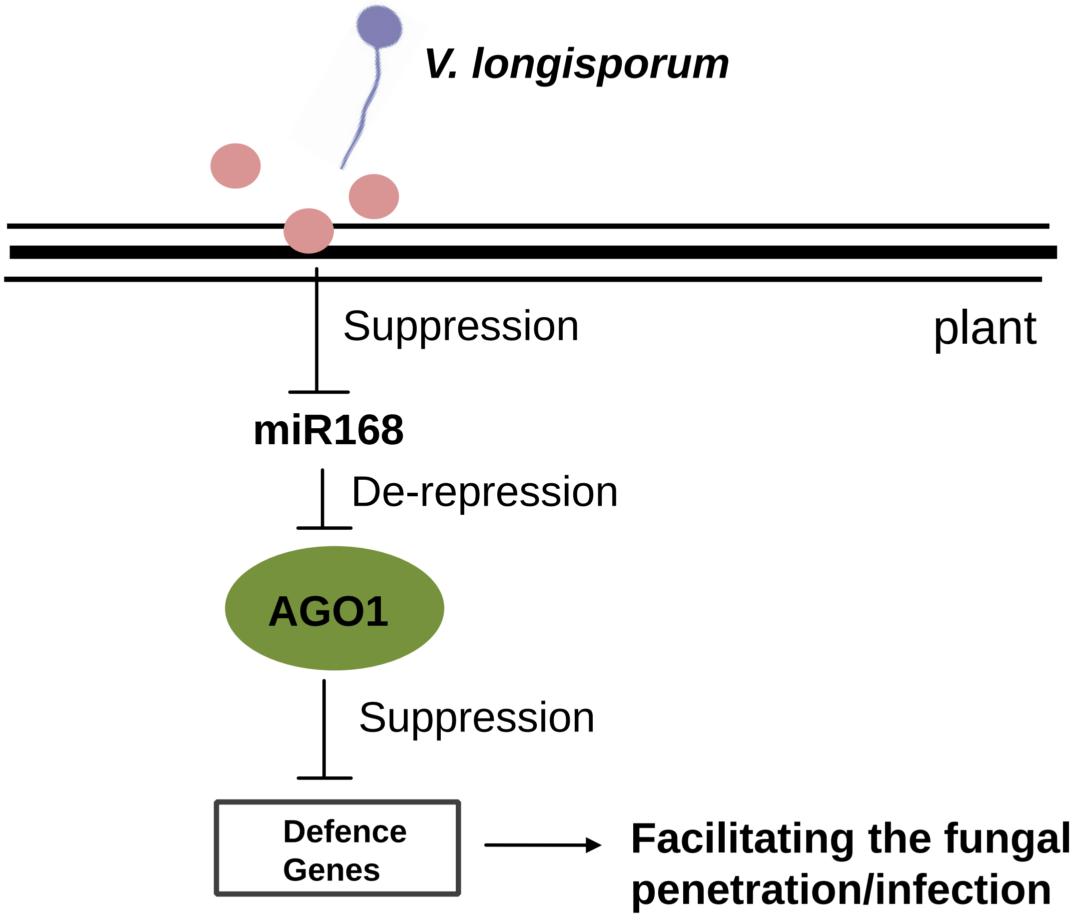
Infection experiments with Arabidopsis *mir168* and *ago1* knockout mutants



Determination of fungal DNA in infected plants

- Disease symptoms: *mir168b > Col-0 > ago1-t*
- Fungi biomass*: mir168b > Col-0 > ago1-t*

A possible action model of miR168-AGO interference



V. longisporum

Suppression

plant

miR168

De-repression

AGO1

Suppression

Defence Genes

Facilitating the fungal penetration/infection

V. longisporum-triggered down-regulation of miR168 leads to de-repression of AGO1 and suppression of plant defence mechanisms, consequently facilitating fungal infection process.

Summary

1. Combination of AA- and CC- genome sequence facilities genome-wide miRNA identification in *B. napus*.

2. 893 miRNAs (360 conserved and 533 specific) were identified from *B. napus,* and A- and C- genome donate an equal number of miRNAs to *B. napus*

3. 137 syntenic pairs of conserved miRNAs were identified. Most of conserved miRNAs from AA and CC genome are homological and most of *B. napus* specific miRNAs are AA-/CC- genome specific.

4. Our results provide further data for understanding the evolution of *B. napus* polyploid genome.

5. More than 90% miRNAs have predicted targets and 20 miRNAs targets were validated.

6. A set of miRNAs (especially, miR168, miR160, miR164, miR167, miR1885) is highly responsive to *V. longisporum* infection.

7. miR168- AGO1 mediates *V. longisporum* susceptibility in *Arabidopsis* by suppression of plant defence-related genes.

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Thank for your attention!