Supplemental Data. Li et al. (2013). Plant Cell 10.1105/tpc.113.112235

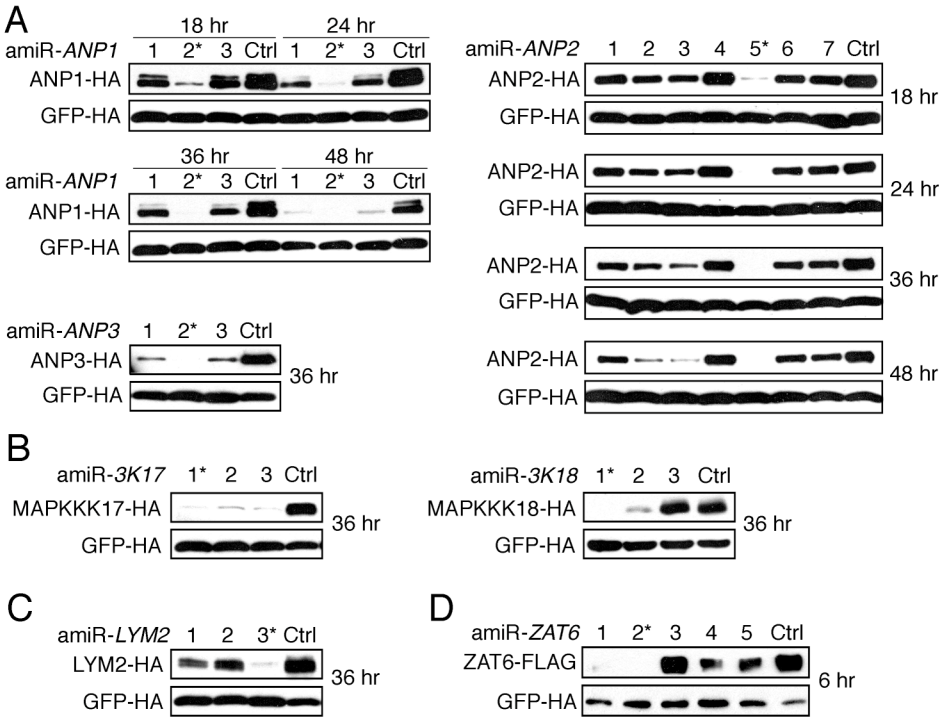
Comprehensive Protein-Based Artificial microRNA Screens for Effective Gene Silencing in Plants

Jian-Feng Li, Hoo Sun Chung, Yajie Niu, Jenifer Bush, Matthew McCormack and Jen Sheen

SUPPLEMENTAL DATA

Supplemental Figure 1. WMD-predicted amiRNA candidates for single gene silencing. A, amiR-*MEKK1*s for silencing *Arabidopsis MEKK1*. B, amiR-*PDS3*s for silencing *Arabidopsis PDS3*. C, amiR-*GFP*s for silencing *GFP*. WMD ranks putative amiRNA candidates by sequence complementarity and hybridization energy, and colors them in green, yellow/orange and red for favorable, intermediate and unfavorable candidates, respectively. The total numbers of amiRNA candidates in individual categories are summarized at the bottom for

each target gene. Selected amiRNA candidates (with sequences underlined) for the ETPamir screen generally should have different target sites within the target gene and should have no potential off-targets. In addition, amiRNA candidates targeting the coding region are preferred over those targeting the UTRs due to easier DNA construction for epitope-tagged target protein expression. The amiRNA sequence (column 1), the hybridization energy of the amiRNA to a perfect complement (column 2), the target gene (column 3), the hybridization energy of the amiRNA to the target site within the target gene (column 4), and the name of the selected amiRNA candidate or the reason for non-selection (column 5) are shown for individual amiRNA candidates from the predicted top candidate to the last selected candidate. The most efficient amiRNA identified by the screen is labeled by a red dot.



Supplemental Figure 2. ETPamir screens of optimal amiRNAs for other single gene silencing in *Arabidopsis*. A, ETPamir screens of optimal amiRNAs silencing individual genes, *ANP1*, *ANP2* and *ANP3*, of the *MAPKKK ANP* family. B, ETPamir screens of optimal amiRNAs silencing individual genes, *MAPKKK17* and *MAPKKK18*, of the *MAPKKK17/18* family. C, ETPamir screen of optimal amiRNA silencing *LYM2* that encodes a plasma membrane protein with unclear function. D, ETPamir screen of optimal amiRNA silencing *ZAT6* that encodes a zinc finger transcription factor. Note that the screen was conducted for only 6 hr due to the short half life (about 10 min) of ZAT6 protein. The numerical order of each amiRNA was based on the high-to-low WMD ranking. The most efficient amiRNAs are marked by asterisks. Five independent repeats with GFP-HA as an untargeted internal control obtained similar results.

A

WMD3-Web MicroRNA Designer

Transcript library: TAIR8_cdna_20080412
Target genes: AT1G18080.1, AT1G48630.1, AT3G18130.1
Description: *RACK1a*, *RACK1b*, *RACK1c* multiple silencing
Min. number of included targets: 3
Accepted off-targets: 0
Annoted: 1

TTAGTAACGACCAACAGCCCA	-45.39	AT3G18130	-31.78	AT1G18080	-31.78	AT1G48630	-31.78	amiR-*RACK1*-1
TAGTAACGACCAATACGCCAG	-44.24	AT1G18080	-37.43	AT3G18130	-39.18	AT1G48630	-37.43	the same target as 1
TTAGTAACGACCAATACCCCG	-44.08	AT3G18130	-43.36	AT1G18080	-43.36	AT1G46830	-43.36	the same target as 1
TTAGTAACGACCAATACGCCA	-42.52	AT3G18130	-37.49	AT1G18080	-37.49	AT1G48630	-37.49	the same target as 1
TTAGTAACGACCAACACGCCA	-44.55	AT3G18130	-32.82	AT1G18080	-32.82	AT1G48630	-32.82	the same target as 1
TTAGTAACGACCAATACGCCG	-43.26	AT3G18130	-37.48	AT1G18080	-37.48	AT1G48630	-37.48	the same target as 1
TTAGTAACGACCACTACGCCA	-44.97	AT3G18130	-33.24	AT1G18080	-33.24	AT1G48630	-33.24	the same target as 1
TTAGTAACGACCAATCCCCCG	-46.49	AT3G18130	-38.12	AT1G18080	-38.12	AT1G48630	-38.12	the same target as 1
TAGTAACGACCAATACCCCTG	-45.06	AT1G18080	-41.61	AT3G18130	-41.61	AT1G48630	-41.61	the same target as 1
TAGTAACGACCAATACGCCAT	-42.74	AT3G18130	-36.88	AT1G18080	-37.09	AT1G48630	-37.09	the same target as 1
TAGTAACGACCAATACGCCTG	-44.24	AT1G18080	-35.84	AT3G18130	-35.84	AT1G48630	-35.84	the same target as 1
TTAGTAACGACCAATACCCCA	-43.34	AT3G18130	-43.37	AT1G18080	-43.37	AT1G48630	-43.37	the same target as 1
TAGTAACGACCAATGCCCCAG	-47.25	AT1G18080	-43.34	AT3G18130	-45.09	AT1G48630	-43.34	the same target as 1
TTAGTAACGACCAATCCCCCA	-45.75	AT3G18130	-38.13	AT1G18080	-38.13	AT1G48630	-38.13	the same target as 1
TTAGTAACGACCAATCCCCGA	-45.09	AT3G18130	-38.44	AT1G18080	-38.44	AT1G48630	-38.44	the same target as 1
TAGTAACGACCAATACCCCAG	-45.06	AT1G18080	-43.31	AT3G18130	-45.06	AT1G48630	-43.31	the same target as 1
TCAAAACGAGTCCTTCGGCAA	-44.37	AT3G18130	-39.12	AT1G18080	-38.24	AT1G48630	-37.10	amiR-*RACK1*-2
TCGTACGATCACGACATGCAG	-46.52	AT3G18130	-36.09	AT1G18080	-34.45	AT1G48630	-36.09	amiR-*RACK1*-3
TAGTAACGACCAATACCGCTG	-44.24	AT1G18080	-35.84	AT3G18130	-35.84	AT1G48630	-35.84	potential off-target At1g47950
TTTCAAAACGAGTCCTTCCGC	-43.56	AT3G18130	-32.38	AT1G18080	-43.59	AT1G48630	-32.72	the same target as 2
TAACGAAGTGAGAGTGACCAT	-43.64	AT3G18130	-40.88	AT1G18080	-42.53	AT1G48630	-41.43	amiR-*RACK1*-4

Total: 0 21 38 55

B

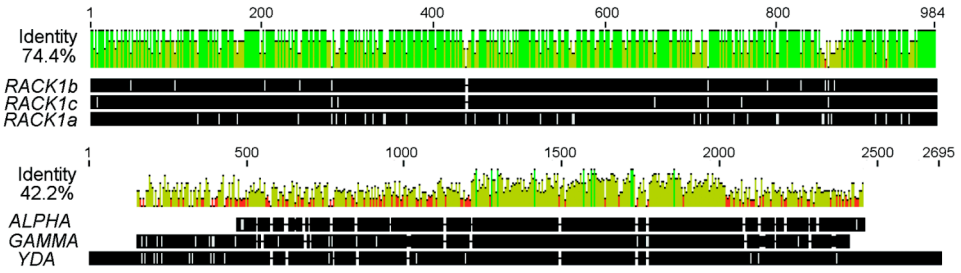
WMD3-Web MicroRNA Designer

Transcript library: TAIR8_cdna_20080412
Target genes: AT1G53570.1, AT1G63700.1, AT5G66850.1
Description: *ALPHA*, *YDA*, *GAMMA* multiple silencing
Min. number of included targets: 3
Accepted off-targets: 0
Annoted: 1

TGTGCCATCCAGTAGGGGCGT	-53.04	AT1G53570	-43.48	AT1G63700	-39.44	AT5G66850	-44.08	amiR-*AYG*-1
TGTGCCATCCAGTAGGCGCTT	-50.47	AT1G53570	-37.71	AT1G63700	-37.23	AT5G66850	-39.22	amiR-*AYG*-2
TGTGCCATCCAGTACCGGCTA	-50.87	AT1G53570	-35.94	AT1G63700	-36.12	AT5G66850	-42.44	potential off-target At2g37650
TGTGCCATCCAGTACCGGCTT	-50.45	AT1G53570	-36.48	AT1G63700	-36.66	AT5G66850	-42.47	amiR-*AYG*-3
TGTGCCATCCAGTACGGGCTT	-50.45	AT1G53570	-40.68	AT1G63700	-40.95	AT5G66850	-49.37	amiR-*AYG*-4

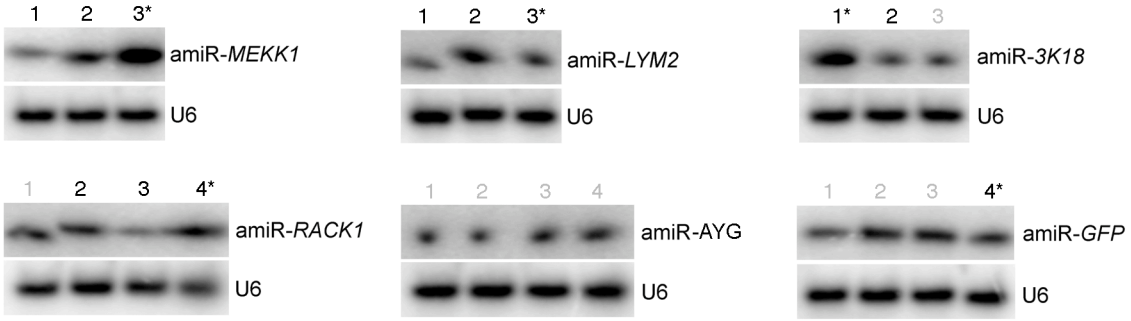
Total: 0 0 2 3

C



Supplemental Figure 3. WMD-predicted amiRNA candidates for multigene silencing. A, amiR-*RACK1*s for silencing the *RACK1* family. B, amiR-AYGs for silencing the *MAPKKK YDA* family. AYG stands for *ALPHA*, *YDA* and *GAMMA*. C, The *YDA* family members have limited sequence identity. Coding sequence alignment of the *RACK1* family (upper panel) or the *YDA* family (lower panel) was conducted by the Geneious program. Identical, similar and distinct

nucleotides are indicated in green, yellow and red, respectively. For A and B, WMD ranks putative amiRNA candidates by sequence complementarity and hybridization energy, and colors them in green, yellow/orange and red for favorable, intermediate and unfavorable candidates, respectively. The total numbers of amiRNA candidates in individual categories are summarized at the bottom for each gene family. Selected amiRNA candidates (with sequences underlined) for the screen generally should have different target sites within the target gene and should have no potential off-targets. The amiRNA sequence (column 1), the hybridization energy of the amiRNA to a perfect complement (column 2), the target gene (column 3, 5 and 7), the corresponding hybridization energy of the amiRNA to the target site within each target gene (column 4, 6 and 8), and the name of the selected amiRNA candidate or the reason for non-selection (column 9) are shown for individual amiRNA candidates from the predicted top candidate to the last selected candidate. The optimal amiRNA candidate identified by the screen is labeled by a red dot.



Supplemental Figure 4. RNA blot analysis of amiRNA expression. The amiRNAs were expressed in *Arabidopsis* mesophyll protoplasts for 6 hr and those targeting the same gene or gene family were blotted onto the same membrane. A mixture (3 or 4 as indicated) of probes were used in RNA blot for each membrane. The small noncoding RNA U6 was used for control hybridization. The optimal amiRNAs are marked by asterisks and ineffective amiRNAs are colored in gray. *3K18* stands for *MAPKKK18*, and AYG for *ALPHA*, *YDA* and *GAMMA*.

A

WMD3-Web MicroRNA Designer

Transcript library: TAIR8_cdna_20080412
Target genes: AT1G53570.1
Description: *ALPHA* single silencing
Min. number of included targets: 1
Accepted off-targets: 0
Annoted: 1

TACTGTTTTATACATGGTCAC	-38.75	AT1G53570	-36.14	have target site at 3' UTR
TACACACAACTAAGGTCGCAT	-43.19	AT1G53570	-36.29	have target site at 3' UTR
TATAGTACAACCCAAACTCAA	-39.11	AT1G53570	-36.83	potential off-target At3g61370
TTTAATAGCACACATTTCCCC	-40.63	AT1G53570	-34.20	amiR-*ALPHA*-1
TAATTATCATGGCTGAGTCTC	-41.79	AT1G53570	-37.03	amiR-*ALPHA*-2
TGTAGAAGACTCTAAGCGCAT	-43.75	AT1G53570	-37.42	amiR-*ALPHA*-3

Total: 91 96 137 537

B

WMD3-Web MicroRNA Designer

Transcript library: TAIR8_cdna_20080412
Target genes: AT1G63700.1
Description: *YDA* single silencing
Min. number of included targets: 1
Accepted off-targets: 0
Annoted: 1

TAAACAGTACATCCAAGACTA	-39.49	AT1G63700	-37.84	amiR-*YDA*-1
TAGTTAACTGTAGTTTGTCTC	-38.54	AT1G63700	-34.18	amiR-*YDA*-2
TTACGAATGGCATTCTGACGA	-42.82	AT1G63700	-34.97	amiR-*YDA*-3

Total: 94 88 130 604

C

WMD3-Web MicroRNA Designer

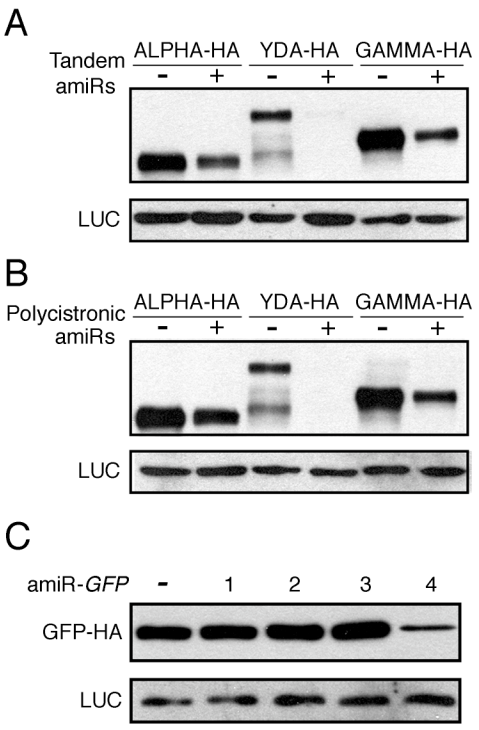
Transcript library: TAIR8_cdna_20080412
Target genes: AT5G66850.1
Description: *GAMMA* single silencing
Min. number of included targets: 1
Accepted off-targets: 0
Annoted: 1

TAATACGTATAGAAGTAGCTG	-38.46	AT5G66850	-35.22	have target site at 3' UTR
TTATTTGTTTATTGGACCCGG	-39.46	AT5G66850	-36.16	amiR-*GAMMA*-1
TTAAAACGAAACAAGCCGCGA	-40.56	AT5G66850	-33.66	amiR-*GAMMA*-2
TTTGATATGACGAATTCGCCG	-41.17	AT5G66850	-34.61	potential off-target At4g14210
TCTAAATACGTACATTGGCAT	-38.71	AT5G66850	-35.80	have target site at 3' UTR
TATTATCGGTACTAAAGGCAG	-40.46	AT5G66850	-39.12	amiR-*GAMMA*-3

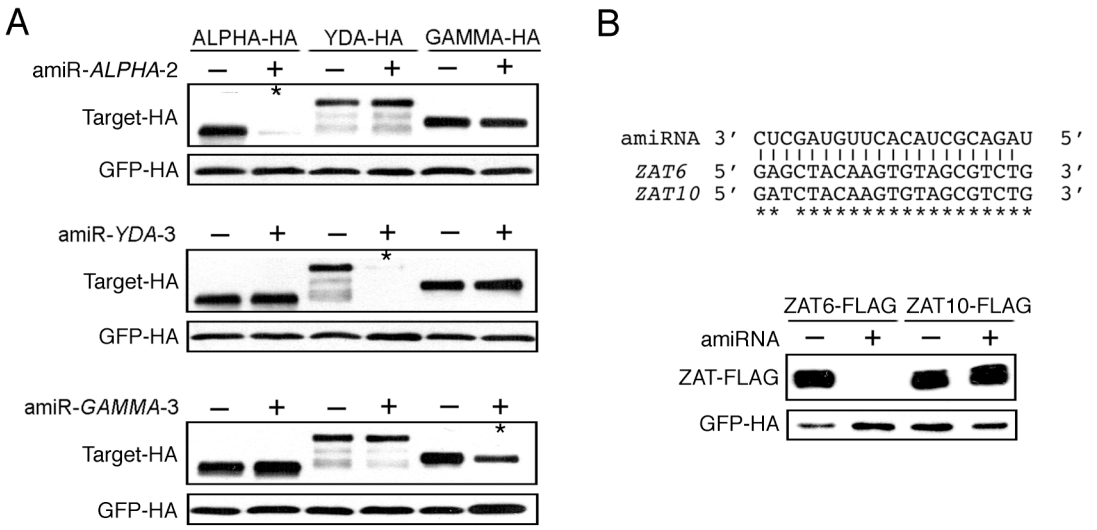
Total: 70 89 137 555

Supplemental Figure 5. WMD-predicted amiRNA candidates for silencing individual members of the *MAPKKK YDA* family. A, amiR-*ALPHA*s for silencing *ALPHA*. B, amiR-*YDA*s for silencing *YDA*. C, amiR-*GAMMA*s for silencing *GAMMA*. The total numbers of amiRNA candidates in individual categories are summarized at the bottom for each target gene. Selected amiRNA candidates (with sequences underlined) for the ETPamir screen generally should have different target sites within the target gene and should have no potential off-targets. In addition, amiRNA candidates targeting the coding region are preferred over those targeting the UTRs due to easier DNA construction for epitope-tagged target protein expression. The amiRNA sequence (column 1), the hybridization energy of the amiRNA to a perfect complement (column 2), the target gene

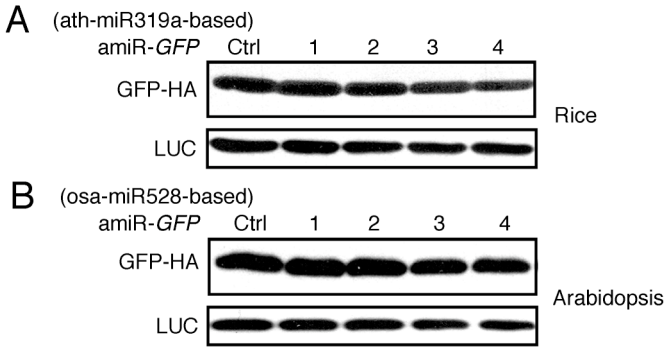
(column 3), the hybridization energy of the amiRNA to the target site within the target gene (column 4), and the name of the selected amiRNA candidate or the reason for non-selection (column 5) are shown for individual amiRNA candidates from the predicted top candidate to the last selected candidate. The most efficient amiRNA identified by the screen is labeled by a red dot.



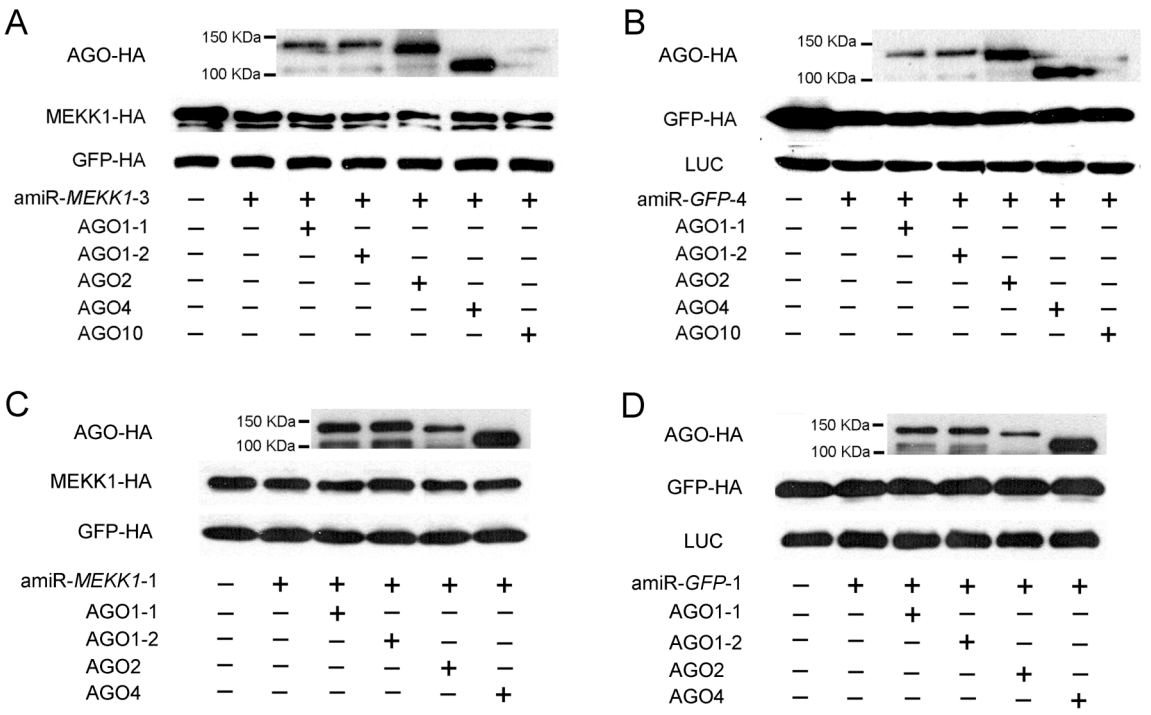
Supplemental Figure 6. *In planta* validation of amiRNA-mediated gene silencing by tobacco leaf agro-infiltration. A, Silencing of the *Arabidopsis MAPKKK YDA* family members (*ALPHA*, *YDA* and *GAMMA*) by tandem optimal amiRNAs. The tandem strategy expressed amiR-*YDA*-3, amiR-*GAMMA*-3 and amiR-*ALPHA*-2 in separate transcripts. B, Silencing of the *Arabidopsis YDA* family members by polycistronic optimal amiRNAs. The polycistronic strategy produced amiR-*YDA*-3, amiR-*GAMMA*-3 and amiR-*ALPHA*-2 from a single transcript. C, Silencing of *GFP* by amiR-*GFP*s. Cocktail of Agrobacteria cells with final OD_{600} of 0.08 for those expressing amiRNA(s) and 0.02 for those expressing the target gene or firefly luciferase (LUC, internal control) were used for tobacco leaf infiltration. Target protein expression were examined by SDS-PAGE and immunoblot analysis at 72 hr post infiltration.



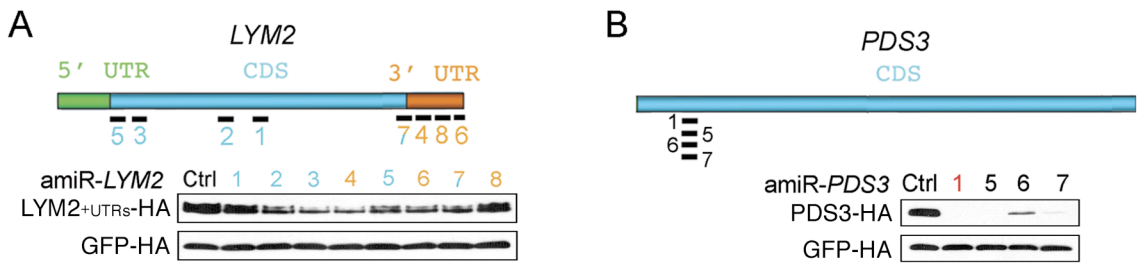
Supplemental Figure 7. ETPamir screens reveal high specificity of gene silencing by plant amiRNAs. A, The optimal amiRNAs for individual genes (*ALPHA*, *YDA* and *GAMMA*) of the *MAPKKK YDA* family only silence one specific member in the family. Efficient gene silencing is indicated by asterisk for each optimal amiRNA. Expression of *ALPHA*, *YDA* and *GAMMA* was induced by 1 hr heat shock pulse after 3 hr constitutive expression of the indicated amiRNA. B, amiR-*ZAT6* does not silence *ZAT10*. *ZAT10*, a closely related homolog of *ZAT6*, possesses a nearly identical sequence to the amiRNA target sequence in *ZAT6* as shown by the sequence alignment, and both proteins have equally short half lives. Individual *ZAT*s were constitutively expressed with or without amiR-*ZAT6* for 6 hr. GFP-HA served as a loading control. Four independent repeats were conducted for A and three for B with similar observations.



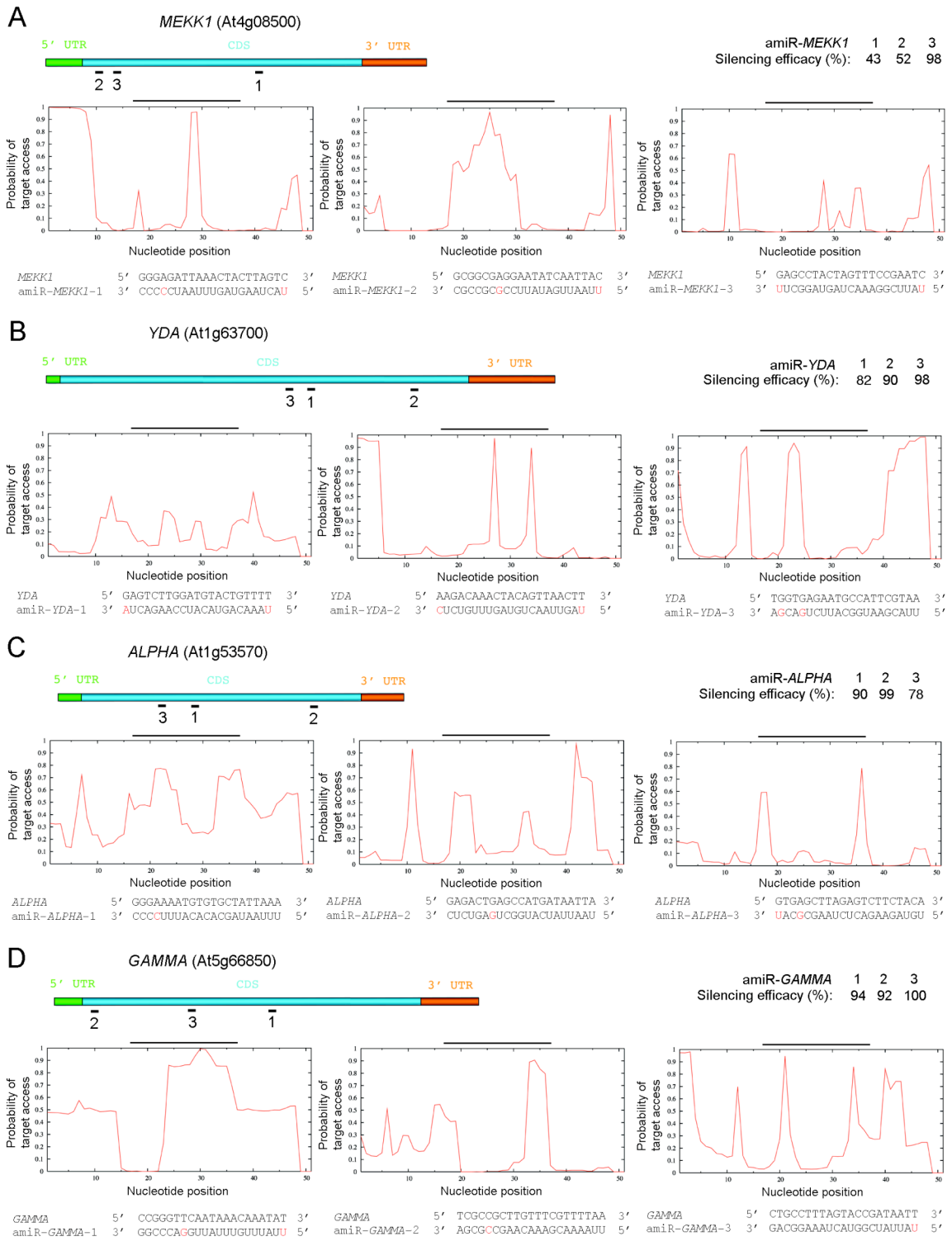
Supplemental Figure 8. Limited cross-species activity of *Arabidopsis* miR319a-derived amiRNA and rice miR528-derived amiRNA. A, *Arabidopsis* miR319a-derived amiR-*GFP*-4 has weak activity in rice protoplasts. B, Rice miR528-derived amiR-*GFP*-4 has weak activity in *Arabidopsis* protoplasts. Luciferase (LUC) served as a loading control. Three independent repeats were conducted for A and two for B with similar results.

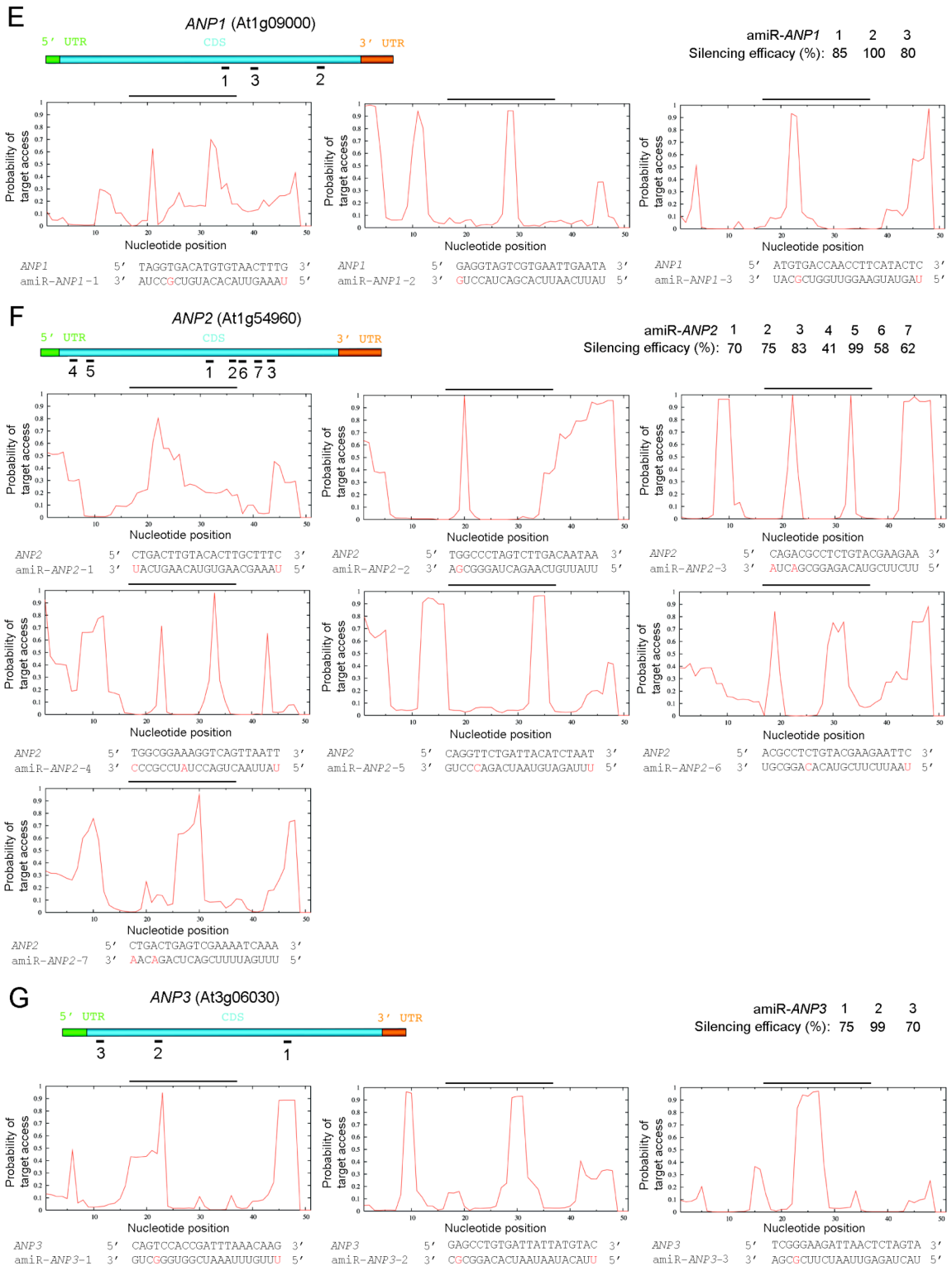


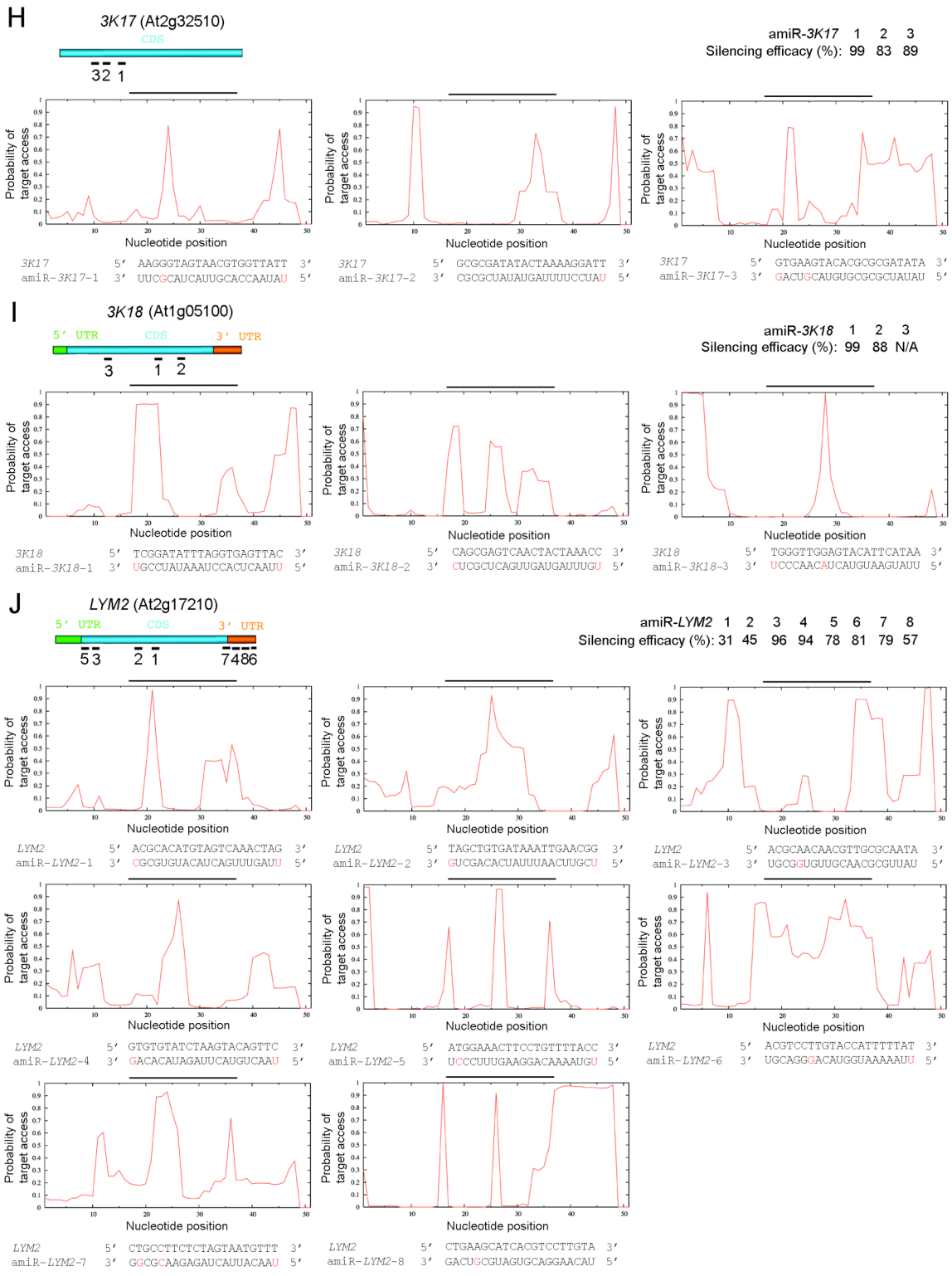
Supplemental Figure 9. Unlimited Argonaute activity in *Arabidopsis* mesophyll protoplasts. A, Co-expression of *Argonaute* (*AGO*) genes can not significantly enhance *MEKK1* silencing by the optimal amiR-*MEKK1*-3. B, Co-expression of *AGO* genes can not significantly enhance *GFP* silencing by the optimal amiR-*GFP*-4. C, Co-expression of *Argonaute* (*AGO*) genes can not significantly enhance *MEKK1* silencing by the suboptimal amiR-*MEKK1*-1. D, Co-expression of *AGO* genes can not significantly enhance *GFP* silencing by the inactive amiR-*GFP*-1. *AGO1-1* and *AGO1-2* are two alternatively spliced isoforms cloned from *Arabidopsis* mesophyll protoplasts. Expression of all constructs was driven by the *35S* promoter for 8 hr. At this time point, obvious but not complete protein silencing was observed for optimal amiRNAs with or without *AGO* co-expression. Two independent repeats with GFP-HA or LUC as internal control were conducted with similar results.

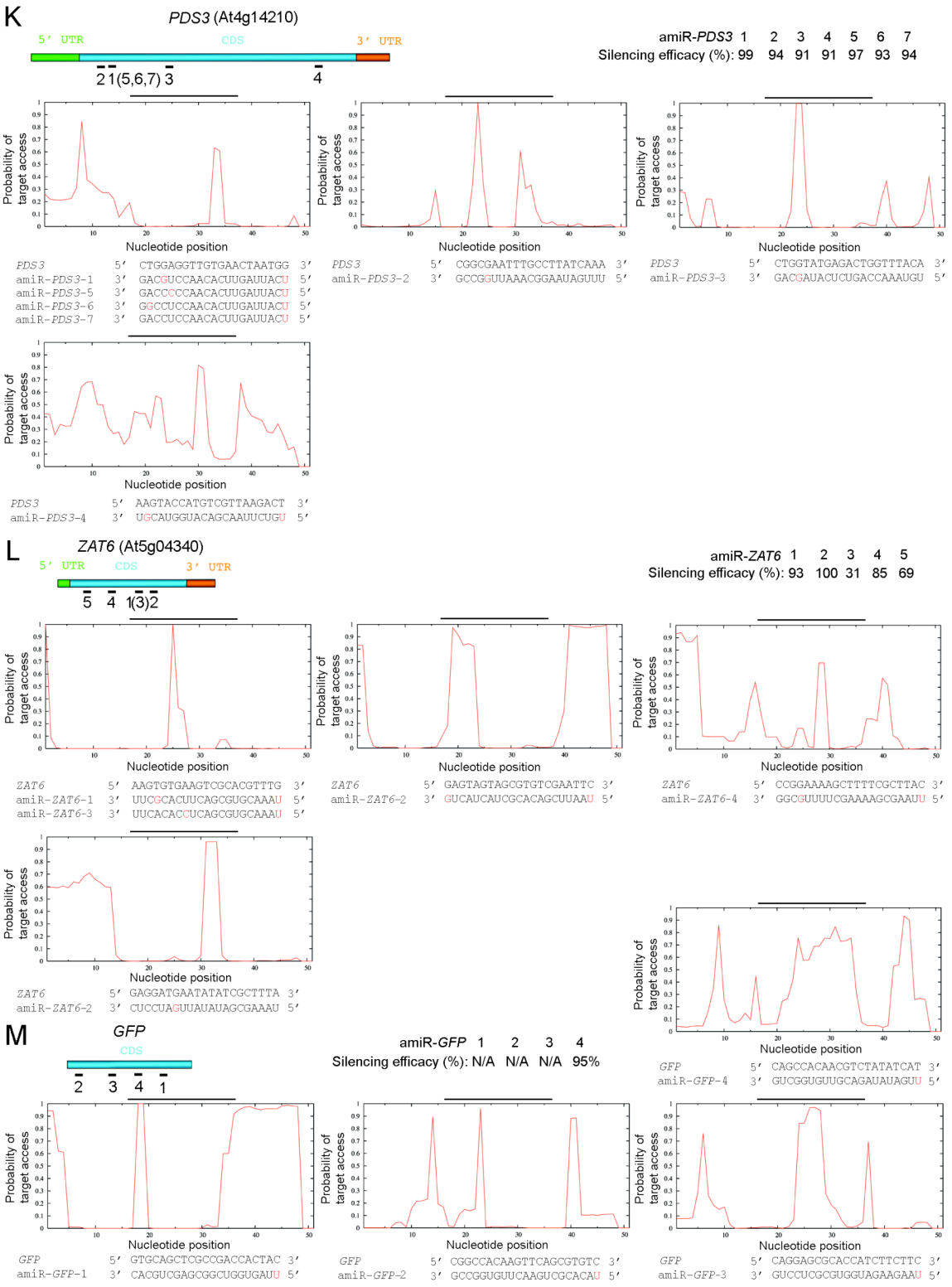


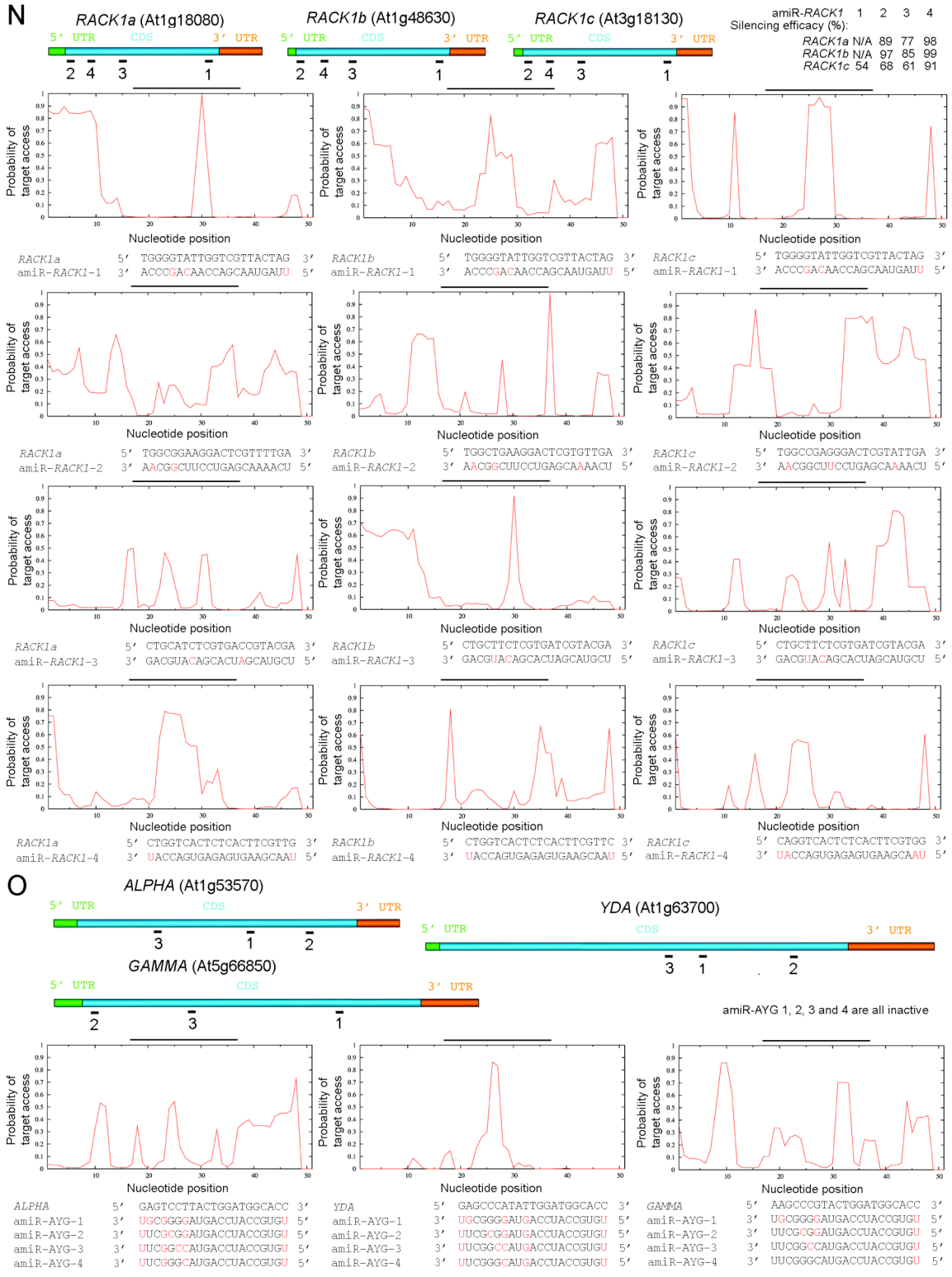
Supplemental Figure 10. No tight correlation between the 3' UTR targeting or WMD ranking of an amiRNA and its efficacy. A, Targeting the 3' UTR does not guarantee plant amiRNA an optimal efficacy as in the cases of animal miRNAs. To test the efficacy of UTR-targeting amiR-*LYM2*s, both 5' UTR and 3' UTR of *LYM2* were constructed into the *LYM2-HA* expression cassette. Individual amiR-*LYM2*s are colored according to the target site location. Note that the target site of amiR-*LYM2*-7 spans from the coding region to the 3' UTR. B, amiRNA candidates with low WMD ranking can have similarly high silencing efficiency. amiR-*PDS3*-5/6/7 shares the same target site with the most efficient amiR-*PDS3*-1 (red) but ranks low in the WMD output list as shown in Supplemental Figure 1B. Expression of *LYM2*$_{+UTRs}$ and *PDS3* was induced by 1 hr heat shock pulse after 3 hr constitutive expression of the indicated amiRNA. Heat shock inducible GFP-HA served as a loading control. All experiments were repeated four times with similar results.

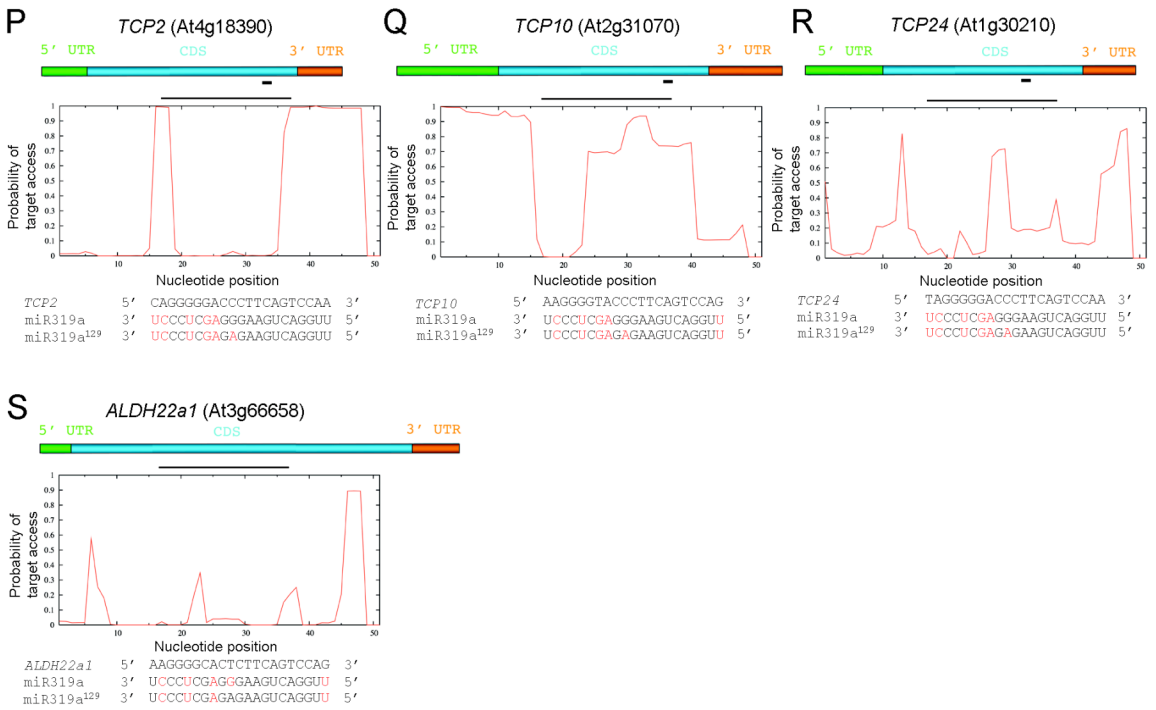

A
MEKK1 (At4g08500)
5′ UTR
CDS
3′ UTR
2 3
1
amiR-MEKK1 1 2 3
Silencing efficacy (%): 43 52 98
Probability of target access
Nucleotide position
MEKK1 5′ GGGAGATTAAACTACTTAGTC 3′
amiR-MEKK1-1 3′ CCCCCUAAUUUGAUGAAUCAU 5′
MEKK1 5′ GCGGCGAGGAATATCAATTAC 3′
amiR-MEKK1-2 3′ CGCCGCGCCUUAUAGUUAAUU 5′
MEKK1 5′ GAGCCTACTAGTTTCCGAATC 3′
amiR-MEKK1-3 3′ UUCGGAUGAUCAAAGGCUUAU 5′
B
YDA (At1g63700)
5′ UTR
CDS
3′ UTR
3 1
2
amiR-YDA 1 2 3
Silencing efficacy (%): 82 90 98
Probability of target access
Nucleotide position
YDA 5′ GAGTCTTGGATGTACTGTTTT 3′
amiR-YDA-1 3′ AUCAGAACCUACAUGACAAAU 5′
YDA 5′ AAGACAAACTACAGTTAACTT 3′
amiR-YDA-2 3′ CUCUGUUUGAUGUCAAUUGAU 5′
YDA 5′ TGGTGAGAATGCCATTCGTAA 3′
amiR-YDA-3 3′ AGCAGUCUUACGGUAAGCAUU 5′
C
ALPHA (At1g53570)
5′ UTR
CDS
3′ UTR
3 1
2
amiR-ALPHA 1 2 3
Silencing efficacy (%): 90 99 78
Probability of target access
Nucleotide position
ALPHA 5′ GGGAAAATGTGTGCTATTAAA 3′
amiR-ALPHA-1 3′ CCCCUUUACACACGAUAAUUU 5′
ALPHA 5′ GAGACTGAGCCATGATAATTA 3′
amiR-ALPHA-2 3′ CUCUGAGUCGGUACUAUUAAU 5′
ALPHA 5′ GTGAGCTTAGAGTCTTCTACA 3′
amiR-ALPHA-3 3′ UACGCGAAUCUCAGAAGAUGU 5′
D
GAMMA (At5g66850)
5′ UTR
CDS
3′ UTR
2
3
1
amiR-GAMMA 1 2 3
Silencing efficacy (%): 94 92 100
Probability of target access
Nucleotide position
GAMMA 5′ CCGGGTTCAATAAACAAATAT 3′
amiR-GAMMA-1 3′ GGCCCAGGUUAUUUGUUUAUU 5′
GAMMA 5′ TCGCCGCTTGTTTCGTTTTAA 3′
amiR-GAMMA-2 3′ AGCGCCGAACAAAGCAAAAUU 5′
GAMMA 5′ CTGCCTTTAGTACCGATAATT 3′
amiR-GAMMA-3 3′ GACGGAAAUCAUGGCUAUUAU 5′


E
ANP1 (At1g09000)
5′ UTR
CDS
3′ UTR
amiR-ANP1 1 2 3
Silencing efficacy (%): 85 100 80
Probability of target access
Nucleotide position
ANP1 5′ TAGGTGACATGTGTAACTTTG 3′
amiR-ANP1-1 3′ AUCCGCUGUACACAUUGAAAU 5′
ANP1 5′ GAGGTAGTCGTGAATTGAATA 3′
amiR-ANP1-2 3′ GUCCAUCAGCACUUAACUUAU 5′
ANP1 5′ ATGTGACCAACCTTCATACTC 3′
amiR-ANP1-3 3′ UACGCUGGUUGGAAGUAUGAU 5′
F
ANP2 (At1g54960)
5′ UTR
CDS
3′ UTR
amiR-ANP2 1 2 3 4 5 6 7
Silencing efficacy (%): 70 75 83 41 99 58 62
Probability of target access
Nucleotide position
ANP2 5′ CTGACTTGTACACTTGCTTTC 3′
amiR-ANP2-1 3′ UACUGAACAUGUGAACGAAAU 5′
ANP2 5′ TGGCCCTAGTCTTGACAATAA 3′
amiR-ANP2-2 3′ AGCGGGAUCAGAACUGUUAUU 5′
ANP2 5′ CAGACGCCTCTGTACGAAGAA 3′
amiR-ANP2-3 3′ AUCAGCGGAGACAUGCUUCUU 5′
ANP2 5′ TGGCGGAAAGGTCAGTTAATT 3′
amiR-ANP2-4 3′ CCCGCCUAUCCAGUCAAUUAU 5′
ANP2 5′ CAGGTTCTGATTACATCTAAT 3′
amiR-ANP2-5 3′ GUCCCAGACUAAUGUAGAUUU 5′
ANP2 5′ ACGCCTCTGTACGAAGAATTC 3′
amiR-ANP2-6 3′ UGCGGACACAUGCUUCUUAAU 5′
ANP2 5′ CTGACTGAGTCGAAAATCAAA 3′
amiR-ANP2-7 3′ AACAGACUCAGCUUUUAGUUU 5′
G
ANP3 (At3g06030)
5′ UTR
CDS
3′ UTR
amiR-ANP3 1 2 3
Silencing efficacy (%): 75 99 70
Probability of target access
Nucleotide position
ANP3 5′ CAGTCCACCGATTTAAACAAG 3′
amiR-ANP3-1 3′ GUCGGGUGGCUAAAUUUGUUU 5′
ANP3 5′ GAGCCTGTGATTATTATGTAC 3′
amiR-ANP3-2 3′ CGCGGACACUAAUAAUACAUU 5′
ANP3 5′ TCGGGAAGATTAACTCTAGTA 3′
amiR-ANP3-3 3′ AGCGCUUCUAAUUGAGAUCAU 5′


H
3K17 (At2g32510)
CDS
32 1
amiR-3K17 1 2 3
Silencing efficacy (%): 99 83 89
Probability of target access
Nucleotide position
3K17 5′ AAGGGTAGTAACGTGGTTATT 3′
amiR-3K17-1 3′ UUCGCAUCAUUGCACCAAUAU 5′
3K17 5′ GCGCGATATACTAAAAGGATT 3′
amiR-3K17-2 3′ CGCGCUAUAUGAUUUUCCUAU 5′
3K17 5′ GTGAAGTACACGCGCGATATA 3′
amiR-3K17-3 3′ GACUGCAUGUGCGCGCUAUAU 5′
I
3K18 (At1g05100)
5′ UTR
CDS
3′ UTR
3 1 2
amiR-3K18 1 2 3
Silencing efficacy (%): 99 88 N/A
3K18 5′ TCGGATATTTAGGTGAGTTAC 3′
amiR-3K18-1 3′ UGCCUAUAAAUCCACUCAAUU 5′
3K18 5′ CAGCGAGTCAACTACTAAACC 3′
amiR-3K18-2 3′ CUCGCUCAGUUGAUGAUUUGU 5′
3K18 5′ TGGGTTGGAGTACATTCATAA 3′
amiR-3K18-3 3′ UCCCAACAUCAUGUAAGUAUU 5′
J
LYM2 (At2g17210)
5′ UTR
CDS
3′ UTR
53 2 1 7486
amiR-LYM2 1 2 3 4 5 6 7 8
Silencing efficacy (%): 31 45 96 94 78 81 79 57
LYM2 5′ ACGCACATGTAGTCAAACTAG 3′
amiR-LYM2-1 3′ CGCGUGUACAUCAGUUUGAUU 5′
LYM2 5′ TAGCTGTGATAAATTGAACGG 3′
amiR-LYM2-2 3′ GUCGACACUAUUUAACUUGCU 5′
LYM2 5′ ACGCAACAACGTTGCGCAATA 3′
amiR-LYM2-3 3′ UGCGGUGUUGCAACGCGUUAU 5′
LYM2 5′ GTGTGTATCTAAGTACAGTTC 3′
amiR-LYM2-4 3′ GACACAUAGAUUCAUGUCAAU 5′
LYM2 5′ ATGGAAACTTCCTGTTTTACC 3′
amiR-LYM2-5 3′ UCCCUUUGAAGGACAAAAUGU 5′
LYM2 5′ ACGTCCTTGTACCATTTTTAT 3′
amiR-LYM2-6 3′ UGCAGGGACAUGGUAAAAAUU 5′
LYM2 5′ CTGCCTTCTCTAGTAATGTTT 3′
amiR-LYM2-7 3′ GGCGCAAGAGAUCAUUACAAU 5′
LYM2 5′ CTGAAGCATCACGTCCTTGTA 3′
amiR-LYM2-8 3′ GACUGCGUAGUGCAGGAACAU 5′


K
PDS3 (At4g14210)
5′ UTR
CDS
3′ UTR
2 1(5,6,7) 3
4
amiR-PDS3 1 2 3 4 5 6 7
Silencing efficacy (%): 99 94 91 91 97 93 94
Probability of target access
Nucleotide position
PDS3 5′ CTGGAGGTTGTGAACTAATGG 3′
amiR-PDS3-1 3′ GACGUCCAACACUUGAUUACU 5′
amiR-PDS3-5 3′ GACCCCCAACACUUGAUUACU 5′
amiR-PDS3-6 3′ GGCCUCCAACACUUGAUUACU 5′
amiR-PDS3-7 3′ GACCUCCAACACUUGAUUACU 5′
PDS3 5′ CGGCGAATTTGCCTTATCAAA 3′
amiR-PDS3-2 3′ GCCGGUUAAACGGAAUAGUUU 5′
PDS3 5′ CTGGTATGAGACTGGTTTACA 3′
amiR-PDS3-3 3′ GACGAUACUCUGACCAAAUGU 5′
PDS3 5′ AAGTACCATGTCGTTAAGACT 3′
amiR-PDS3-4 3′ UGCAUGGUACAGCAAUUCUGU 5′
L
ZAT6 (At5g04340)
5′ UTR
CDS
3′ UTR
5 4 1(3)2
amiR-ZAT6 1 2 3 4 5
Silencing efficacy (%): 93 100 31 85 69
ZAT6 5′ AAGTGTGAAGTCGCACGTTTG 3′
amiR-ZAT6-1 3′ UUCGCACUUCAGCGUGCAAAU 5′
amiR-ZAT6-3 3′ UUCACACCUCAGCGUGCAAAU 5′
ZAT6 5′ GAGTAGTAGCGTGTCGAATTC 3′
amiR-ZAT6-2 3′ GUCAUCAUCGCACAGCUUAAU 5′
ZAT6 5′ CCGGAAAAGCTTTTCGCTTAC 3′
amiR-ZAT6-4 3′ GGCGUUUUCGAAAAGCGAAUU 5′
ZAT6 5′ GAGGATGAATATATCGCTTTA 3′
amiR-ZAT6-2 3′ CUCCUAGUUAUAUAGCGAAAU 5′
M
GFP
CDS
2 3 4 1
amiR-GFP 1 2 3 4
Silencing efficacy (%): N/A N/A N/A 95%
GFP 5′ GTGCAGCTCGCCGACCACTAC 3′
amiR-GFP-1 3′ CACGUCGAGCGGCUGGUGAUU 5′
GFP 5′ CGGCCACAAGTTCAGCGTGTC 3′
amiR-GFP-2 3′ GCCGGUGUUCAAGUCGCACAU 5′
GFP 5′ CAGCCACAACGTCTATATCAT 3′
amiR-GFP-4 3′ GUCGGUGUUGCAGAUAUAGUU 5′
GFP 5′ CAGGAGCGCACCATCTTCTTC 3′
amiR-GFP-3 3′ GUCCUCGCGUGGUAGAAGAAU 5′


N
RACK1a (At1g18080)
RACK1b (At1g48630)
RACK1c (At3g18130)
5′ UTR CDS 3′ UTR
amiR-RACK1 1 2 3 4
Silencing efficacy (%):
RACK1a N/A 89 77 98
RACK1b N/A 97 85 99
RACK1c 54 68 61 91
Probability of target access
Nucleotide position
RACK1a 5′ TGGGGTATTGGTCGTTACTAG 3′
amiR-RACK1-1 3′ ACCCGACAACCAGCAAUGAUU 5′
RACK1b 5′ TGGGGTATTGGTCGTTACTAG 3′
amiR-RACK1-1 3′ ACCCGACAACCAGCAAUGAUU 5′
RACK1c 5′ TGGGGTATTGGTCGTTACTAG 3′
amiR-RACK1-1 3′ ACCCGACAACCAGCAAUGAUU 5′
RACK1a 5′ TGGCGGAAGGACTCGTTTTGA 3′
amiR-RACK1-2 3′ AACGGCUUCCUGAGCAAAACU 5′
RACK1b 5′ TGGCTGAAGGACTCGTGTTGA 3′
amiR-RACK1-2 3′ AACGGCUUCCUGAGCAAAACU 5′
RACK1c 5′ TGGCCGAGGGACTCGTATTGA 3′
amiR-RACK1-2 3′ AACGGCUUCCUGAGCAAAACU 5′
RACK1a 5′ CTGCATCTCGTGACCGTACGA 3′
amiR-RACK1-3 3′ GACGUACAGCACUAGCAUGCU 5′
RACK1b 5′ CTGCTTCTCGTGATCGTACGA 3′
amiR-RACK1-3 3′ GACGUACAGCACUAGCAUGCU 5′
RACK1c 5′ CTGCTTCTCGTGATCGTACGA 3′
amiR-RACK1-3 3′ GACGUACAGCACUAGCAUGCU 5′
RACK1a 5′ CTGGTCACTCTCACTTCGTTG 3′
amiR-RACK1-4 3′ UACCAGUGAGAGUGAAGCAAU 5′
RACK1b 5′ CTGGTCACTCTCACTTCGTTC 3′
amiR-RACK1-4 3′ UACCAGUGAGAGUGAAGCAAU 5′
RACK1c 5′ CAGGTCACTCTCACTTCGTGG 3′
amiR-RACK1-4 3′ UACCAGUGAGAGUGAAGCAAU 5′
O
ALPHA (At1g53570)
YDA (At1g63700)
GAMMA (At5g66850)
amiR-AYG 1, 2, 3 and 4 are all inactive
ALPHA 5′ GAGTCCTTACTGGATGGCACC 3′
amiR-AYG-1 3′ UGCGGGGAUGACCUACCGUGU 5′
amiR-AYG-2 3′ UUCGCGGAUGACCUACCGUGU 5′
amiR-AYG-3 3′ UUCGGCCAUGACCUACCGUGU 5′
amiR-AYG-4 3′ UUCGGGCAUGACCUACCGUGU 5′
YDA 5′ GAGCCCATATTGGATGGCACC 3′
amiR-AYG-1 3′ UGCGGGGAUGACCUACCGUGU 5′
amiR-AYG-2 3′ UUCGCGGAUGACCUACCGUGU 5′
amiR-AYG-3 3′ UUCGGCCAUGACCUACCGUGU 5′
amiR-AYG-4 3′ UUCGGGCAUGACCUACCGUGU 5′
GAMMA 5′ AAGCCCGTACTGGATGGCACC 3′
amiR-AYG-1 3′ UGCGGGGAUGACCUACCGUGU 5′
amiR-AYG-2 3′ UUCGCGGAUGACCUACCGUGU 5′
amiR-AYG-3 3′ UUCGGCCAUGACCUACCGUGU 5′
amiR-AYG-4 3′ UUCGGGCAUGACCUACCGUGU 5′



Supplemental Figure 11. Visual summary of amiRNA/miRNA target site location, predicted target accessibility and target complementarity. A, *MEKK1*; B, *YDA*; C, *ALPHA*; D, *GAMMA*; E, *ANP1*; F, *ANP2*; G, *ANP3*; H, *MAPKKK17* (*3K17*); I, *MAPKKK18* (*3K18*); J, *LYM2*; K, *PDS3*; L, *ZAT6*; M, *GFP*; N, the *RACK1* family; O, the *MAPKKK YDA* family; P, *TCP2*; Q, *TCP10*; R, *TCP24*; S, *ALDH22a1*. The target site accessibility was predicted by the Sfold algorithm based on a 51-nt target region (the 21-nt target sequence plus 17-nt upstream and 13-nt downstream sequences). The horizontal line on top of each target accessibility plot marks the exact position of target site in the target region. Mismatches between amiRNA/miRNA and its target sequence(s) are highlighted in red. The silencing efficiency of individual amiRNAs determined in ETPamir screens is also summarized.

Supplemental Table 1. Summary of amiRNAs for silencing the *MAPKKK YDA* family

amiRNA	Target gene	Hybridization energy (kcal/mol)	Mismatch number and position	Target site/CDS (5' to 3')	WMD prediction	Silencin gefficien cy
AYG-1[a]	At5g66850	-44.08 (83.11%)[b]	3 (1, 15, 20)	1524-1544 /2151	Less favorable[c]	ND[d]
	At1g53570	-43.48 (81.98%)	5 (1, 15, 18, 20, 21)	1125-1145 /1827		ND
	At1g63700	-39.44 (74.36%)	5 (1, 12, 15, 20, 21)	1683-1703 /2652		ND
AYG-2	At5g66850	-39.22 (77.71%)	3 (1, 15, 17)	1524-1544 /2151	Less favorable	ND
	At1g53570	-37.71 (74.72%)	5 (1, 15, 17, 18, 21)	1125-1145 /1827		ND
	At1g63700	-37.23 (73.77%)	5 (1, 12, 15, 17, 21)	1683-1703 /2652		ND
AYG-3	At5g66850	-42.47 (84.18%)	2 (1, 16)	1524-1544 /2151	Unfavorable	ND
	At1g53570	-36.48 (72.31%)	5 (1, 15, 16, 18, 21)	1125-1145 /1827		ND
	At1g63700	-36.66 (72.67%)	5 (1, 12, 15, 16, 21)	1683-1703 /2652		ND
AYG-4	At5g66850	-49.37 (97.86%)	1 (1)	1524-1544 /2151	Unfavorable	ND
	At1g53570	-40.68 (80.63%)	4 (1, 15, 18, 21)	1125-1145 /1827		ND
	At1g63700	-40.95 (81.17%)	4 (1, 12, 15, 21)	1683-1703 /2652		ND

[a]The numerical order of each amiRNA is based on the high-to-low WMD ranking.
[b]The number in parentheses = hybridization energy of the amiRNA to the target site/that of the amiRNA to a perfect complement ×100%.
[c]WMD categorizes predicted amiRNA candidates based on sequence complementarity and hybridization energy.
[d]ND: No detectable gene silencing.

Supplemental Table 2. Predicted natural target genes for *Arabidopsis* miR319a

Prediction server/database	Reference	Predicted target genes (total gene number)
WMD	Ossowski et al., 2008	***TCP2***, ***TCP3****, ***TCP4****, ***TCP10***, ***TCP24***, ***MYB33***, ***MYB65***, *MYB104*, *ALDH22a1* (9)
TAPIR	Bonnet et al., 2010	***TCP2***, ***TCP3***, ***TCP4***, ***TCP10***, ***TCP24***, ***MYB33***, ***MYB65***, *MYB104*, *ALDH22a1* (9)
UEA Plant sRNA toolkit	Moxon et al., 2008	***TCP4***, ***TCP10***, ***MYB33***, ***MYB65***, *MYB104*, At5g67090 (encoding subtilase family protein) (6)
RNAhybrid	Alves-Junior et al., 2009	***TCP4***, ***MYB33***, ***MYB65***, *MYB104*, At5g67090 (encoding subtilase family protein) (5)
starBase	Yang et al., 2011	***TCP2***, ***TCP24***, ***MYB33***, ***MYB65*** (4)
psRNATarget	Dai and Zhao, 2011	***MYB33***, *MYB104*, At5g67090 (encoding subtilase family protein) (3)
Plant microRNA database (PMRD)	Zhang et al., 2010	***MYB33***, ***MYB65***, *MYB104* (3)

**TCP3* and *TCP4* are predicted by WMD as miR319a target genes only if the first 20 nucleotides of miR319a are input for target search (personal communication with Rebecca Schwab)
Genes with name in bold have been previously predicted by Jones-Rhoades et al. (2004) as miR319a targets without using the above servers, and have been experimentally validated by Palatnik et al. (2003 and 2007) as natural targets. *MYB104* has also been predicted by Jones-Rhoades et al. (2004) as miR319a target without using the above servers.
Genes with name underlined are investigated in this work, among which *TCP2*, *TCP10* and *TCP24* but not *ALDH22a1* are validated as natural targets of miR319a.
Target genes are all predicted using the default setting in each server/database.

Supplemental Table 3. Recombinant plasmids constructed during this study

No.	Plasmid name	Expression cassette		Usage
		Promoter	Gene/amiRNA	
1	amiR-MEK-1	*35S*	amiR-*MEKK1*-1	Protoplast expression
2	amiR-MEK-2	*35S*	amiR-*MEKK1*-2	Protoplast expression
3	amiR-MEK-3	*35S*	amiR-*MEKK1*-3	Protoplast expression
4	amiR-y-1	*35S*	amiR-*YDA*-1	Protoplast expression
5	amiR-y-2	*35S*	amiR-*YDA*-2	Protoplast expression
6	amiR-y-3	*35S*	amiR-*YDA*-3	Protoplast expression
7	amiR-a-1	*35S*	amiR-*ALPHA*-1	Protoplast expression
8	amiR-a-2	*35S*	amiR-*ALPHA*-2	Protoplast expression
9	amiR-a-3	*35S*	amiR-*ALPHA*-3	Protoplast expression
10	amiR-r-1	*35S*	amiR-*GAMMA*-1	Protoplast expression
11	amiR-r-2	*35S*	amiR-*GAMMA*-2	Protoplast expression
12	amiR-r-3	*35S*	amiR-*GAMMA*-3	Protoplast expression
13	amiR-AP1-1	*35S*	amiR-*ANP1*-1	Protoplast expression
14	amiR-AP1-2	*35S*	amiR-*ANP1*-2	Protoplast expression
15	amiR-AP1-3	*35S*	amiR-*ANP1*-3	Protoplast expression
16	amiR-AP2-1	*35S*	amiR-*ANP2*-1	Protoplast expression
17	amiR-AP2-2	*35S*	amiR-*ANP2*-2	Protoplast expression
18	amiR-AP2-3	*35S*	amiR-*ANP2*-3	Protoplast expression
19	amiR-AP2-4	*35S*	amiR-*ANP2*-4	Protoplast expression
20	amiR-AP2-5	*35S*	amiR-*ANP2*-5	Protoplast expression
21	amiR-AP2-6	*35S*	amiR-*ANP2*-6	Protoplast expression
22	amiR-AP2-7	*35S*	amiR-*ANP2*-7	Protoplast expression
23	amiR-AP3-1	*35S*	amiR-*ANP3*-1	Protoplast expression
24	amiR-AP3-2	*35S*	amiR-*ANP3*-2	Protoplast expression
25	amiR-AP3-3	*35S*	amiR-*ANP3*-3	Protoplast expression
26	amiR-17-1	*35S*	amiR-*3K17*-1	Protoplast expression
27	amiR-17-2	*35S*	amiR-*3K17*-2	Protoplast expression
28	amiR-17-3	*35S*	amiR-*3K17*-3	Protoplast expression
29	amiR-18-1	*35S*	amiR-*3K18*-1	Protoplast expression
30	amiR-18-2	*35S*	amiR-*3K18*-2	Protoplast expression
31	amiR-18-3	*35S*	amiR-*3K18*-3	Protoplast expression
32	amiR-LM2-1	*35S*	amiR-*LYM2*-1	Protoplast expression
33	amiR-LM2-2	*35S*	amiR-*LYM2*-2	Protoplast expression
34	amiR-LM2-3	*35S*	amiR-*LYM2*-3	Protoplast expression
35	amiR-LM2-4	*35S*	amiR-*LYM2*-4	Protoplast expression
36	amiR-LM2-5	*35S*	amiR-*LYM2*-5	Protoplast expression
37	amiR-LM2-6	*35S*	amiR-*LYM2*-6	Protoplast expression
38	amiR-LM2-7	*35S*	amiR-*LYM2*-7	Protoplast expression
39	amiR-LM2-8	*35S*	amiR-*LYM2*-8	Protoplast expression
40	amiR-PDS-1	*35S*	amiR-*PDS3*-1	Protoplast expression
41	amiR-PDS-2	*35S*	amiR-*PDS3*-2	Protoplast expression
42	amiR-PDS-3	*35S*	amiR-*PDS3*-3	Protoplast expression
43	amiR-PDS-4	*35S*	amiR-*PDS3*-4	Protoplast expression
44	amiR-PDS-5	*35S*	amiR-*PDS3*-5	Protoplast expression
45	amiR-PDS-6	*35S*	amiR-*PDS3*-6	Protoplast expression
46	amiR-PDS-7	*35S*	amiR-*PDS3*-7	Protoplast expression
47	amiR-ZT6-1	*35S*	amiR-*ZAT6*-1	Protoplast expression
48	amiR-ZT6-2	*35S*	amiR-*ZAT6*-2	Protoplast expression
49	amiR-ZT6-3	*35S*	amiR-*ZAT6*-3	Protoplast expression
50	amiR-ZT6-4	*35S*	amiR-*ZAT6*-4	Protoplast expression
51	amiR-ZT6-5	*35S*	amiR-*ZAT6*-5	Protoplast expression
52	amiR-GFP-1(miR319)	*35S*	amiR-*GFP*-1	Protoplast expression
53	amiR-GFP-2(miR319)	*35S*	amiR-*GFP*-2	Protoplast expression
54	amiR-GFP-3(miR319)	*35S*	amiR-*GFP*-3	Protoplast expression
55	amiR-GFP-4(miR319)	*35S*	amiR-*GFP*-4	Protoplast expression
56	amiR-RK-1	*35S*	amiR-*RACK1*-1	Protoplast expression
57	amiR-RK-2	*35S*	amiR-*RACK1*-2	Protoplast expression
58	amiR-RK-3	*35S*	amiR-*RACK1*-3	Protoplast expression
59	amiR-RK-3	*35S*	amiR-*RACK1*-4	Protoplast expression
60	amiR-AYG-1	*35S*	amiR-*AYG*-1	Protoplast expression
61	amiR-AYG-2	*35S*	amiR-*AYG*-2	Protoplast expression
62	amiR-AYG-3	*35S*	amiR-*AYG*-3	Protoplast expression
63	amiR-AYG-4	*35S*	amiR-*AYG*-4	Protoplast expression
64	miR319a-129	*35S*	miR319a[129]	Protoplast expression
65	HBT-MEKK1-HA	*35S*	*MEKK1*	Protoplast expression
66	HBT-YDA-HA	*35S*	*YDA*	Protoplast expression
67	HBT-ALPHA-HA	*35S*	*ALPHA*	Protoplast expression

68	HBT-GAMMA-HA	*35S*	*GAMMA*	Protoplast expression
69	HBT-ANP1-HA	*35S*	*ANP1*	Protoplast expression
70	HBT-ANP2-HA	*35S*	*ANP2*	Protoplast expression
71	HBT-ANP3-HA	*35S*	*ANP3*	Protoplast expression
72	HBT-17-HA	*35S*	*MAPKKK17*	Protoplast expression
73	HBT-18-HA	*35S*	*MAPKKK18*	Protoplast expression
74	HBT-LYM2-HA	*35S*	*LYM2*	Protoplast expression
75	HBT-PDS3-HA	*35S*	*PDS3*	Protoplast expression
76	HBT-ZAT6-HA	*35S*	*ZAT6*	Protoplast expression
77	HBT-GFP-HA	*35S*	*GFP*	Protoplast expression
78	HBT-RACK1a-HA	*35S*	*RACK1a*	Protoplast expression
79	HBT-RACK1b-HA	*35S*	*RACK1b*	Protoplast expression
80	HBT-RACK1c-HA	*35S*	*RACK1c*	Protoplast expression
81	HBT-AGO1-1-HA	*35S*	*AGO1-1*	Protoplast expression
82	HBT-AGO1-2-HA	*35S*	*AGO1-2*	Protoplast expression
83	HBT-AGO2-HA	*35S*	*AGO2*	Protoplast expression
84	HBT-AGO4-HA	*35S*	*AGO4*	Protoplast expression
85	HBT-AGO10-HA	*35S*	*AGO10*	Protoplast expression
86	HSP-MEKK1-HA	*Heat shock*	*MEKK1*	Protoplast expression
87	HSP-YDA-HA	*Heat shock*	*YDA*	Protoplast expression
88	HSP-ALPHA-HA	*Heat shock*	*ALPHA*	Protoplast expression
89	HSP-GAMMA-HA	*Heat shock*	*GAMMA*	Protoplast expression
90	HSP-ANP1-HA	*Heat shock*	*ANP1*	Protoplast expression
91	HSP-ANP2-HA	*Heat shock*	*ANP2*	Protoplast expression
92	HSP-ANP3-HA	*Heat shock*	*ANP3*	Protoplast expression
93	HSP-17-HA	*Heat shock*	*MAPKKK17*	Protoplast expression
94	HSP-18-HA	*Heat shock*	*MAPKKK18*	Protoplast expression
95	HSP-LYM2-HA	*Heat shock*	*LYM2*	Protoplast expression
96	HSP-LYM2UTR-HA	*Heat shock*	*LYM2* with *5' UTR & 3' UTR*	Protoplast expression
97	HSP-PDS3-HA	*Heat shock*	*PDS3*	Protoplast expression
98	HSP-GFP-HA	*Heat shock*	*GFP*	Protoplast expression
99	HSP-RACK1a-HA	*Heat shock*	*RACK1a*	Protoplast expression
100	HSP-RACK1b-HA	*Heat shock*	*RACK1b*	Protoplast expression
101	HSP-RACK1c-HA	*Heat shock*	*RACK1c*	Protoplast expression
102	HSP-TCP2-HA	*Heat shock*	*TCP2*	Protoplast expression
103	HSP-TCP10-HA	*Heat shock*	*TCP10*	Protoplast expression
104	HSP-TCP24-HA	*Heat shock*	*TCP24*	Protoplast expression
105	HSP-TCP20-HA	*Heat shock*	*TCP20*	Protoplast expression
106	HSP-ALDH22a1-HA	*Heat shock*	*ALDH22a1*	Protoplast expression
107	3SUMO-Target-y-HA	*35S*	*3XSUMO$_{AA}$-Target$_{amiR}$*-YDA-*3*	Protoplast expression
108	2SUMO-Target-r-HA	*35S*	*2XSUMO$_{AA}$-Target$_{amiR}$*-GAMMA-*3*	Protoplast expression
109	SUMO-Target-a-HA	*35S*	*SUMO$_{AA}$-Target$_{amiR}$*-ALPHA-*3*	Protoplast expression
110	amiR-Poly-AYG	*35S*	amiR-*YDA*-3, amiR-*GAMMA*-3, amiR-*ALPHA*-2	Protoplast expression
111	amiR-Tandem-AYG	*35S* *35S* *35S*	amiR-*YDA*-3 amiR-*GAMMA*-3 amiR-*ALPHA*-2	Protoplast expression
112	pFGC-amiR-Poly	*35S*	amiR-*YDA*-3, amiR-*GAMMA*-3, amiR-*ALPHA*-2	Tobacco leaf infiltration
113	pFGC-amiR-Tandem	*35S* *35S* *35S*	amiR-*YDA*-3 amiR-*GAMMA*-3 amiR-*ALPHA*-2	Tobacco leaf infiltration
114	pFGC-YDA-HA	*35S*	*YDA*	Tobacco leaf infiltration
115	pFGC-ALPHA-HA	*35S*	*ALPHA*	Tobacco leaf infiltration
116	pFGC-GAMMA-HA	*35S*	*GAMMA*	Tobacco leaf infiltration
117	pCB302-GFP-HA	*35S*	*GFP*	Tobacco leaf infiltration
118	pCB302-LUC	*35S*	Firefly luciferase	Tobacco leaf infiltration
119	pCB302-amiR-GFP-1	*35S*	amiR-*GFP*-1	Tobacco leaf infiltration
120	pCB302-amiR-GFP-2	*35S*	amiR-*GFP*-2	Tobacco leaf infiltration
121	pCB302-amiR-GFP-3	*35S*	amiR-*GFP*-3	Tobacco leaf infiltration
122	pCB302-amiR-GFP-4	*35S*	amiR-*GFP*-4	Tobacco leaf infiltration
123	pCB302-amiR-MEK-1	*35S*	amiR-*MEKK1*-1	Transgenic expression
124	pCB302-amiR-MEK-3	*35S*	amiR-*MEKK1*-3	Transgenic expression
125	pCB302-amiR-PDS-1	*35S*	amiR-*PDS3*-1	Transgenic expression
126	pCB302-amiR-PDS-4	*35S*	amiR-*PDS3*-4	Transgenic expression
127	pCB302-amiR-RK-1	*35S*	amiR-*RACK1*-1	Transgenic expression
128	pCB302-amiR-RK-4	*35S*	amiR-*RACK1*-4	Transgenic expression
129	pFGC-amiR-MEKK1	*35S* *Estradiol-inducible*	*GFP-Target$_{amiR}$*-MEKK1-*3* amiR-*MEKK1*-3	Transgenic expression

130	amiR-GFP-1(miR528)	*35S*	amiR-*GFP*-1	Protoplast expression
131	amiR-GFP-2(miR528)	*35S*	amiR-*GFP*-2	Protoplast expression
132	amiR-GFP-3(miR528)	*35S*	amiR-*GFP*-3	Protoplast expression
133	amiR-GFP-4(miR528)	*35S*	amiR-*GFP*-4	Protoplast expression

Supplemental Table 4. Primers used for qPCR in this study

Gene	Primer name	Sequence (5' to 3')	Amplicon position
MEKK1	MEKK1-qPCR-5-F	ACTGGACGAAGAGGAGGAGA	Upstream of the amiR-*MEKK1*-3 target site
	MEKK1-qPCR-5-R	TCAACGAAGAAGTCGAAACG	
	MEKK1-qPCR-c-F	TACGACGCAGCTTCATGTTC	Spanning the amiR-*MEKK1*-3 target site
	MEKK1-qPCR-c-R	AGCCAAATCATCAGGACCAG	
	MEKK1-qPCR-3-F	CCGAAAGGATAGTGATGGCTATGG	Downstream of the amiR-*MEKK1*-3 target site
	MEKK1-qPCR-3-R	GATCCTAAACAGGGCTTGAACGG	
YDA	YDA-qPCR-5-F	TGAAAGTGGGGAGATGTGTG	Upstream of the amiR-*YDA*-3 target site
	YDA-qPCR-5-R	CGAACCACCGGAGACATACT	
	YDA-qPCR-c-F	AGTATGTCTCCGGTGGTTCG	Spanning the amiR-*YDA*-3 target site
	YDA-qPCR-c-R	CCATCCCAAAATCAGCAACT	
	YDA-qPCR-3-F	GAGCACCATGAGATCACTGGAC	Downstream of the amiR-*YDA*-3 target site
	YDA-qPCR-3-R	TCCGAGTCTAAGCACGGAAGAC	
ALPHA	ALPHA-qPCR-5-F	TGGGATGGCCAAACATGTAACAG	Upstream of the amiR-*ALPHA*-2 target site
	ALPHA-qPCR-5-F	GATATCGACTGCATGAGTGTAGCC	
	ALPHA-qPCR-c-F	TCACTGCCTACAAGGGAACC	Spanning the amiR-*ALPHA*-2 target site
	ALPHA-qPCR-c-F	GGTGAGGAGGTGACAGGAAA	
	ALPHA-qPCR-3-F	TAAAGAGCCCGAGCAGAGAA	Downstream of the amiR-*ALPHA*-2 target site
	ALPHA-qPCR-3-F	CACTGTCTTGCCCAGGAAAT	
GAMMA	GAMMA-qPCR-5-F	CTTCCGCAAATCTCGTTCTC	Upstream of the amiR-*GAMMA*-3 target site
	GAMMA-qPCR-5-F	CCGATCCCGATCCTGATTAC	
	GAMMA-qPCR-c-F	TGCTCCAGATATGCCACTTG	Spanning the amiR-*GAMMA*-3 target site
	GAMMA-qPCR-c-F	AACGGTGAAGATGGTCTGCT	
	GAMMA-qPCR-3-F	AGCGACCAACCGCATCTATGTTG	Downstream of the amiR-*GAMMA*-3 target site
	GAMMA-qPCR-3-F	TCCCTTCTACTGCTTGGTTCCG	
ANP1	ANP1-qPCR-5-F	AGCTGAGTTGGCTACGATGACTGGT	Upstream of the amiR-*ANP1*-2 target site
	ANP1-qPCR-5-F	CCGACGCTCCATATGTCAGCAGAG	
	ANP1-qPCR-c-F	AGAGGACACTGCTCGTGGTT	Spanning the amiR-*ANP1*-2 target site
	ANP1-qPCR-c-F	CGTGCTGATACACCATCCTG	
	ANP1-qPCR-3-F	GGGGTCTCGTTGTTGACACT	Downstream of the amiR-*ANP1*-2 target site
	ANP1-qPCR-3-F	CCTCTGTCTCTTGGGGATGA	
ANP2	ANP2-qPCR-5-F	AATCCCAGTCACCTCCGAAT	Upstream of the amiR-*ANP2*-5 target site
	ANP2-qPCR-5-F	GAACCTGTTTAACGGCGAGA	
	ANP2-qPCR-c-F	TCTCGCCGTTAAACAGGTTC	Spanning the amiR-*ANP2*-5 target site
	ANP2-qPCR-c-F	TCATCTTCCCTCACCGTACC	
	ANP2-qPCR-3-F	AGCAATGCAAGTGCTGGTGCTG	Downstream of the amiR-*ANP2*-5 target site
	ANP2-qPCR-3-F	TCCCTGCTTGCCGTGTAATCTCTC	
ANP3	ANP3-qPCR-5-F	GGTGGAGGAAAGGGGAATTA	Upstream of the amiR-*ANP3*-2 target site
	ANP3-qPCR-5-F	TTCCTCAAGCTCTCGGATGT	
	ANP3-qPCR-c-F	TCTTTCACATCCGAACATCG	Spanning the amiR-*ANP3*-2 target site
	ANP3-qPCR-c-F	GATGCATGATCCCATTGTTG	
	ANP3-qPCR-3-F	AGAGCTTGAGAGGCATCGAGAG	Downstream of the amiR-*ANP3*-2 target site
	ANP3-qPCR-3-F	AATGGAGTCTTCCCTCCTGCAC	
MAP KKK17	3K17-qPCR-5-F	GTATGGAACGTTGACCGATGCG	Upstream of the amiR-*3K17*-1 target site
	3K17-qPCR-5-F	ATCGCGCGTGTACTTCACTACC	
	3K17-qPCR-c-F	CTCGAAAGGAATCGTGCATT	Spanning the amiR-*3K17*-1 target site
	3K17-qPCR-c-F	CCACCTCTGGAGCCATAAAA	
	3K17-qPCR-3-F	GTGTGTTGGTGGTTTGGATGGG	Downstream of the amiR-*3K17*-1 target site
	3K17-qPCR-3-F	TTCCTTCACACCTCGCTCTCAC	
MAP KKK18	3K18-qPCR-5-F	AAGCAACGTGTTGGTCGGAGAG	Upstream of the amiR-*3K18*-1 target site
	3K18-qPCR-5-F	CGGTTCAACCCATTTCGCACAC	
	3K18-qPCR-c-F	GAGATGGTTACCGGGTCTCA	Spanning the amiR-*3K18*-1 target site
	3K18-qPCR-c-F	CATCTCTCCGTCGCTTCTTT	
	3K18-qPCR-3-F	TCAAGCTGGTGGGAATGTCACG	Downstream of the amiR-*3K18*-1 target site
	3K18-qPCR-3-F	TGGACTCCACCACAACATGACC	
LYM2	LYM2-qPCR-5-F	CGTTCCAGACTATGCTTACCC*	Upstream of the amiR-*LYM2*-3 target site
	LYM2-qPCR-5-F	TGCTTGAGTAACCAACGAGAG	
	LYM2-qPCR-c-F	TCAAGCAAGAACGCAACAAC	Spanning the amiR-*LYM2*-3 target site
	LYM2-qPCR-c-F	AGAGCAATGGATTGGGACAC	
	LYM2-qPCR-3-F	GCGTCTATGCTGGTTACTCCAACC	Downstream of the amiR-*LYM2*-3 target site
	LYM2-qPCR-3-F	TCAGGACCAGCAGAATCTGGAC	
PDS3	PDS3-qPCR-5-F	TGGTTGTGTTTGGGAATGTT	Upstream of the amiR-*PDS3*-1 target site
	PDS3-qPCR-5-F	CAGATGAAAGTGCCTCCAAA	
	PDS3-qPCR-c-F	GGTTTTTGGAGGCACTTTCA	Spanning the amiR-*PDS3*-1 target site
	PDS3-qPCR-c-F	TCTCTAGCTCTGGCCTTGGA	
	PDS3-qPCR-3-F	ATTTGCACCAGCAGAGGAAT	Downstream of the amiR-*PDS3*-1 target site
	PDS3-qPCR-3-F	CGACATGGTTCACAGTTTGG	

GFP	GFP-qPCR-5-F GFP-qPCR-5-F	AGGAGCGCACCATCTTCTT TGTAGTTGTACTCCAGCTTGTGC	Downstream of the amiR-*GFP*-4 target site
	GFP-qPCR-c-F GFP-qPCR-c-F	ACGACGGCAACTACAAGACC ACCTTGATGCCGTTCTTCTG	Upstream of the amiR-*GFP*-4 target site
	GFP-qPCR-3-F GFP-qPCR-3-F	TATATCATGGCCGACAAGCA ACTGGGTGCTCAGGTAGTGG	Spanning the amiR-*GFP*-4 target site

*LYM2-qPCR-5-F was designed based on the HA tag coding sequence, which was inserted behind the coding sequence of the signal peptide of LYM2 (amino acids 1-23) and was located upstream of the amiR-*LYM2*-3 target site within *LYM2*.

Supplemental Table 5. Sequences of amiRNA/miRNAs tested during this study

No.	amiRNA name	Target gene	amiRNA sequence (5' to 3')
1	*MEKK1*-1	At4g08500	UACUAAGUAGUUUAAUCCCCC
2	*MEKK1*-2	At4g08500	UUAAUUGAUAUUCCGCGCCGC
3	*MEKK1*-3	At4g08500	UAUUCGGAAACUAGUAGGCUU
4	*YDA*-1	At1g63700	UAAACAGUACAUCCAAGACUA
5	*YDA*-2	At1g63700	UAGUUAACUGUAGUUUGUCUC
6	*YDA*-3	At1g63700	UUACGAAUGGCAUUCUGACGA
7	*ALPHA*-1	At1g53570	UUUAAUAGCACACAUUUCCCC
8	*ALPHA*-2	At1g53570	UAAUUAUCAUGGCUGAGUCUC
9	*ALPHA*-3	At1g53570	UGUAGAAGACUCUAAGCGCAU
10	*GAMMA*-1	At5g66850	UUAUUUGUUUAUUGGACCCGG
11	*GAMMA*-2	At5g66850	UUAAAACGAAACAAGCCGCGA
12	*GAMMA*-3	At5g66850	UAUUAUCGGUACUAAAGGCAG
13	*ANP1*-1	At1g09000	UAAAGUUACACAUGUCGCCUA
14	*ANP1*-2	At1g09000	UAUUCAAUUCACGACUACCUG
15	*ANP1*-3	At1g09000	UAGUAUGAAGGUUGGUCGCAU
16	*ANP2*-1	At1g54960	UAAAGCAAGUGUACAAGUCAU
17	*ANP2*-2	At1g54960	UUAUUGUCAAGACUAGGGCGA
18	*ANP2*-3	At1g54960	UUCUUCGUACAGAGGCGACUA
19	*ANP2*-4	At1g54960	UAUUAACUGACCUAUCCGCCC
20	*ANP2*-5	At1g54960	UUUAGAUGUAAUCAGACCCUG
21	*ANP2*-6	At1g54960	UAAUUCUUCGUACACAGGCGU
22	*ANP2*-7	At1g54960	UUUGAUUUUCGACUCAGACAA
23	*ANP3*-1	At3g06030	UUUGUUUAAAUCGGUGGGCUG
24	*ANP3*-2	At3g06030	UUACAUAAUAAUCACAGGCGC
25	*ANP3*-3	At3g06030	UACUAGAGUUAAUCUUCGCGA
26	*3K17*-1	At2g32510	UAUAACCACGUUACUACGCUU
27	*3K17*-2	At2g32510	UAUCCUUUUAGUAUAUCGCGC
28	*3K17*-3	At2g32510	UAUAUCGCGCGUGUACGUCAG
29	*3K18*-1	At1g05100	UUAACUCACCUAAAUAUCCGU
30	*3K18*-2	At1g05100	UGUUUAGUAGUUGACUCGCUC
31	*3K18*-3	At1g05100	UUAUGAAUGUACUACAACCCU
32	*LYM2*-1	At2g17120	UUAGUUUGACUACAUGUGCGC
33	*LYM2*-2	At2g17120	UCGUUCAAUUUAUCACAGCUG
34	*LYM2*-3	At2g17120	UAUUGCGCAACGUUGUGGCGU
35	*LYM2-4*	At2g17120	UAACUGUACUUAGAUACACAG
36	*LYM2-5*	At2g17120	UGUAAAACAGGAAGUUUCCCU
37	*LYM2-6*	At2g17120	UUAAAAAUGGUACAGGGACGU
38	*LYM2-7*	At2g17120	UAACAUUACUAGAGAACGCGG
39	*LYM2-8*	At2g17120	UACAAGGACGUGAUGCGUCAG
40	*PDS3*-1	At4g14210	UCAUUAGUUCACAACCUGCAG
41	*PDS3*-2	At4g14210	UUUGAUAAGGCAAAUUGGCCG
42	*PDS3*-3	At4g14210	UGUAAACCAGUCUCAUAGCAG
43	*PDS3*-4	At4g14210	UGUCUUAACGACAUGGUACGU
44	*PDS3*-5	At4g14210	UCAUUAGUUCACAACCCCCAG
45	*PDS3*-6	At4g14210	UCAUUAGUUCACAACCUCCGG
46	*PDS3*-7	At4g14210	UCAUUAGUUCACAACCUCCAG
47	*ZAT6*-1	At5g04340	UAAACGUGCGACUUCACGCUU
48	*ZAT6*-2	At5g04340	UAAUUCGACACGCUACUACUG
49	*ZAT6*-3	At5g04340	UAAACGUGCGACUCCACACUU
50	*ZAT6*-4	At5g04340	UUAAGCGAAAAGCUUUUGCGG
51	*ZAT6*-5	At5g04340	UAAAGCGAUAUAUUGAUCCUC
52	*GFP*-1	*GFP*	UUAGUGGUCGGCGAGCUGCAC
53	*GFP*-2	*GFP*	UACACGCUGAACUUGUGGCCG
54	*GFP*-3	*GFP*	UAAGAAGAUGGUGCGCUCCUG
55	*GFP*-4	*GFP*	UUGAUAUAGACGUUGUGGCUG
56	*RACK1*-1	At1g48630 At1g18080 At3g18130	UUAGUAACGACCAACAGCCCA
57	*RACK1*-2	At1g48630 At1g18080 At3g18130	UCAAAACGAGUCCUUCGGCAA
58	*RACK1*-3	At1g48630 At1g18080 At3g18130	UCGUACGAUCACGACAUGCAG
59	*RACK1*-4	At1g48630 At1g18080 At3g18130	UAACGAAGUGAGAGUGACCAU
60	AYG-1	At5g66850 At1g53570 At1g63700	UGUGCCAUCCAGUAGGGGCGU
61	AYG-2	At5g66850 At1g53570 At1g63700	UGUGCCAUCCAGUAGGCGCUU
62	AYG-3	At5g66850 At1g53570 At1g63700	UGUGCCAUCCAGUACCGGCUU
63	AYG-4	At5g66850 At1g53570 At1g63700	UGUGCCAUCCAGUACGGGCUU
64	miR319a	At4g18390 At2g31070 At1g30210	UUGGACUGAAGGGAGCUCCCU
65	miR319a[129]	N/A	UUGGACUGAAGAGAGCUCCCU

SUPPLEMENTAL METHODS 1

Plasmid Construction

All plasmids used in this work are listed in the Supplemental Table 3 and are available upon request. For amiRNA/miRNA expression plasmids (Supplemental Table 3, No.1-64, No.130-133), the precursors for individual amiRNAs or miR319a[129] (Supplemental Table 5) were assembled by a two-step overlapping PCR method using *Arabidopsis* miR319a precursor or rice miR528 precursor as the template according to the instruction from WMD (http://wmd3.weigelworld.org). PCR products of pre-amiRNAs or pre-miR319a[129] were digested by *Bam*HI/*Pst*I and inserted into the same digested HBT vector (Yoo et al., 2007) that contains the *35S* promoter for transient expression in plant protoplasts.

For plasmids constitutively expressing target gene (No.59-74), the full-length coding sequences of target genes were amplified by RT-PCR, digested by *Bam*HI/*Stu*I and inserted into the same digested HBT-2HA vector to express double HA tagged target proteins under the *35S* promoter. For *AGO* expression plasmids (No. 81-85), the coding sequences of *AGO1* (isoforms *AGO1-1* and *AGO1-2*), *AGO2*, *AGO4* and *AGO10* were PCR amplified from *Arabidopsis* mesophyll protoplast cDNAs, digested by *Bam*HI/*Stu*I and inserted into the same digested HBT-2HA vector to express double HA tagged AGO proteins under the *35S* promoter.

For plasmids inducibly expressing target gene (No.86-106), the heat shock protein 18.2 promoter (*HSP*) and the *Nos* terminator were PCR amplified from the template plasmid HSP18.2-LUC-NOS (GenBank ID: EF090413, Yoo et al., 2007), digested by *Eco*RI/*Bam*HI and *Pst*I/*Sma*I, respectively, and inserted into the same sites of the pUC119-RCS vector (Lee et al., 2008) to obtain the pUC119-HSP vector. The full-length coding sequences encoding HA-tagged target proteins were PCR amplified or directly cut out by *Bam*HI/*Pst*I from the HBT-based constitutive expression plasmids, and then inserted into the

*Bam*HI/*Pst*I site of the pUC119-HSP vector. Regarding the plasmid HSP-LYM2UTR-HA (No. 96), the full-length cDNA of *LYM2* (including both UTRs) was amplified by RT-PCR, digested and inserted into the pUC119-HSP vector. The HA tag coding sequence was then introduced behind the coding sequence of the signal peptide of LYM2 (amino acids 1-23) through site-directed mutagenesis.

For plasmids expressing the "SUMO ladder" (No.107-109), the target site of amiR-*YDA*-3, amiR-*GAMMA*-3 or amiR-*ALPHA*-2 was included into the reverse primer to PCR the $SUMO_{AA}$ coding sequence, which expresses the SUMO protein with the last two glycines mutated to alanines to avoid potential post-translational cleavages between SUMO repeats. The PCR products were cloned into the *Bam*HI/*Stu*I site of the HBT-2HA vector. The second and the third $SUMO_{AA}$ coding sequences were sequentially inserted into the *Bam*HI site upstream the first $SUMO_{AA}$ coding sequence and the intended insertion orientation was confirmed by DNA sequencing.

For the plasmid expressing polycistronic amiRNAs (No.110), the second and the third pre-amiRNAs were PCR amplified and digested by *Bam*HI/*Bgl*II and sequentially inserted into the *Bam*HI site upstream the first pre-amiRNA within the HBT plasmid. For the plasmid expressing tandem amiRNAs (Supplemental Table 3, No.111), the first pre-amiRNA expression cassette (*35S:pre-amiRNA:Nos*) was PCR amplified and digested by *Stu*I/*Sma*I and inserted into the *Stu*I site of the pUC119-RCS vector. The PCR products of the second and the third pre-amiRNA expression cassettes were digested by *Stu*I/*Sma*I and sequentially inserted into the *Stu*I site upstream the first pre-amiRNA expression cassette in the pUC119-RCS vector. The correct pre-amiRNA assembling orientation in plasmids No.110 and 111 was confirmed by DNA sequencing. For the binary plasmid expressing polycistronic amiRNAs via tobacco leaf agro-infiltration (No. 112), the polycistronic pre-amiRNAs were extracted from the plasmid No. 110 by *Bam*HI/*Pst*I and inserted into the same cut pUC119-RCS vector containing a *35S* promoter and a *Nos* terminator. The whole expression

cassette was then cut out by *Asc*I and inserted into the same digested pFGC binary vector. For the binary plasmid expressing tandem amiRNAs via tobacco leaf agro-infiltration (No. 113), the tandem pre-amiRNA expression cassettes were cut out from the plasmid No. 111 by *Asc*I and inserted into the same digested pFGC binary vector.

For binary plasmids expressing *YDA*, *ALPHA* or *GAMMA* via tobacco leaf agro-infiltration (No. 114-116), PCR products encoding HA-tagged YDA, ALPHA or GAMMA were cloned into the pUC119-RCS vector containing a *35S* promoter and a *Nos* terminator. The whole expression cassette was then cut out by *Asc*I and inserted into the same digested pFGC binary vector. For binary plasmids expressing *GFP* or firefly luciferase (*LUC*) via tobacco leaf agro-infiltration (No. 117 and 118), the *GFP-HA* coding sequence extracted by *Bam*HI/*Pst*I from the plasmid No. 77 or the *Bam*HI/*Pst*I digested PCR products of LUC was inserted into the same digested pCB302 binary vector (Xiang et al., 1999).

For binary plasmids expressing amiR-*GFP*s via tobacco leaf agro-infiltration (No. 119-122), these pre-amiRNAs were respectively extracted from the plasmids No.52-55 by *Bam*HI/*Pst*I digestion, and then inserted into the same digested pCB302 binary vector.

For binary plasmids expressing amiRNAs in transgenic plants (No.123-128), the pre-amiRNA fragments were extracted from the HBT-based amiRNA expression plasmids by *Bam*HI/*Pst*I digestion and inserted into the same digested pCB302 binary vector. For the binary plasmid expressing the GFP-target sensor (No.129), the target sequence of amiR-*MEKK1*-3 was introduced between *GFP* and the stop codon by PCR. The PCR products were digested by *Xba*I/*Not*I and inserted into the same digested pAN vector (Li and Nebenführ, 2007) containing a *35S* promoter and a *Nos* terminator. The *35S:GFP-Target*$_{amiR\text{-MEKK1-3}}$*:Nos* expression cassette was then removed from the pAN vector by *Sac*I/*Eco*RV digestion and subcloned into the pUC119-RCS vector. The expression cassette was again

extracted by I-*Ceu*I/*Asc*I digestion and inserted into the same digested binary vector pFGC19-XVE-RCS, which expresses the XVE transcription activator (Zuo et al., 2000) under the *35S* promoter, to obtain the intermediate plasmid pFGC-GFP-Target. The *Bam*HI/*Pst*I fragment of pre-amiR-*MEKK1*-3 was inserted between the estradiol-inducible promoter (Curtis and Grossniklaus, 2003) and the *Nos* terminator in a modified pUC119-RCS vector. The pre-amiR-*MEKK1*-3 expression cassette was then extracted by *Asc*I digestion and inserted into the *Asc*I site of the intermediate plasmid pFGC-GFP-Target to obtain pFGC-amiR-MEKK1.

Tobacco Leaf Agro-infiltration

Tobacco leaf agro-infiltration was conducted as previously described (Sparkes et al., 2006) with modifications. Briefly, overnight cultured agrobacteria GV3101 cells harboring correct binary vector were pelleted at 16,000 g for 30 sec and washed once with the infiltration solution (10 mM MES, pH 5.7, 10 mM $MgCl_2$, 100 μM acetosyringone). *Agrobacterium* were resuspended with the infiltration solution and mixed to obtain a final OD_{600} of 0.08 for those expressing amiRNA and 0.02 for those expressing the target gene or firefly luciferase (LUC, internal control). Before infiltration, intended infiltration zones on the underside of the third or fourth leaf of 6 weeks old tobacco plants were labeled with a marker pen. *Agrobacterium* cocktail was gently infiltrated into the marked infiltration zones using a 1-ml syringe without needle. At 72 hr post infiltration, a leaf disc was generated from each infiltration zone using a hole punch (diameter 6 mm). Three leaf discs from three infiltration repeats were powdered in a 1.5 ml microcentrifuge tube in liquid nitrogen bath by a rotor-stator homogenizer and were boiled with 50 μl 1×SDS loading buffer at 95°C for 10 min. Total proteins were subjected to SDS-PAGE and immunoblot analysis using anti-HA (Sigma) or anti-LUC (Santa Cruz Biotechnology) antibodies.

Bioinformatic Analysis

Gene-specific amiRNA candidates were designed by the Web-based MicroRNA Designer (WMD, http://wmd3.weigelworld.org, Schwab et al., 2006) by inputting the gene identification number (for *Arabidopsis* gene) or the coding sequence (for *GFP*). PCR primers for generating a desired amiRNA were also designed through the "Oligo" platform on the WMD website by inputting the amiRNA sequence listed in the Supplemental Table 5. Target site accessibility was predicted by the Sfold server (http://sfold.wadsworth.org, Ding et al., 2004) using a 51-nt target region within the target gene covering the 21-nt target sequence and 17-nt upstream and 13-nt downstream sequences (Kertesz et al., 2007). Natural target genes for plant miRNAs were predicted through the following web servers or databases: TAPIR (http://bioinformatics.psb.ugent.be/webtools/tapir, Bonnet et al., 2010), WMD, UEA plant sRNA toolkit (http://srna-tools.cmp.uea.ac.uk, Moxon et al., 2008), starBase (http://starbase.sysu.edu.cn, Yang et al., 2011), psRNATarget (http://plantgrn.noble.org/psRNATarget, Dai and Zhao, 2011) and Plant microRNA database (PMRD, http://bioinformatics.cau.edu.cn/PMRD, Zhang et al., 2010).

SUPPLEMENTAL REFERENCES

Alves-Junior L, Niemeier S, Hauenschild A, Rehmsmeier M, Merkle T (2009) Comprehensive prediction of novel microRNA targets in Arabidopsis thaliana. Nucleic Acids Res **37:** 4010-4021

Bonnet E, He Y, Billiau K, Van de Peer Y (2010) TAPIR, a web server for the prediction of plant microRNA targets, including target mimics. Bioinformatics **26:** 1566-1568

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133:** 462-469

Dai X, Zhao PX (2011) psRNATarget: A plant small RNA target analysis server. Nucleic Acids Res **39:** W155-W159

Ding Y, Chan CY, Lawrence CE (2004) Sfold web server for statistical folding and rational design of nucleic acids. Nucleic Acids Res **32:** W135-W141

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell **14:** 787-799

Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E (2007) The role of site accessibility in microRNA target recognition. Nat Genet **39:** 1278-1284

Lee LY, Fang MJ, Kuang LY, Gelvin SB (2008) Vectors for multi-color bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. Plant Methods **4:** 24

Li JF, Nebenführ A (2007) Organelle targeting of myosin XI is mediated by two globular tail subdomains with separate cargo binding sites. J Biol Chem **282:** 20593-20602

Moxon S, Schwach F, MacLean D, Dalmay T, Studholme DJ, Moulton V (2008) A toolkit for analysing large-scale plant small RNA datasets. Bioinformatics **24:** 2252-2253

Ossowski S, Schwab R, Weigel D (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. Plant J **53:** 674-690

Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature **425:** 257-263

Palatnik JF, Wollmann H, Schommer C, Schwab R, Boisbouvier J, Rodriguez R, Warthmann N, Allen E, Dezulian T, Huson D, Carrington JC, Weigel D (2007) Sequence and expression differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. Dev Cell **13:** 115-125

Sparkes IA, Runions J, Kearns A, Hawes C (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nat Protocols **1:** 2019-2025

Xiang CB, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. Plant Mol Biol **40:** 711-717

Yang JH, Li JH, Shao P, Zhou H, Chen YQ, Qu LH (2011) starBase: a database for exploring microRNA-mRNA interaction maps from Argonaute CLIP-Seq and Degradome-Seq data. Nucleic Acids Res **39:** D202-D209

Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protocols **2:** 1565-1572

Zhang Z, Yu J, Li D, Zhang Z, Liu F, Zhou X, Wang T, Ling Y, Su Z (2010) PMRD: plant microRNA database. Nucleic Acids Res **38:** D806-D813

Zuo J, Niu QW, Chua NH (2000) An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J **24:** 265-273