Overview on current and future RNAi applications

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Outline

1. RNAi based GM plants to provide resistance against
   - nematodes
   - insects
   - fungi
   - viruses
   - parasitic weeds

2. RNAi based GM plants to manipulate metabolism to improve
   - industrial traits
   - nutritional value

Resistance against nematodes

1. Cyst nematodes

Transgenic *Arabidopsis thaliana* became resistant to *Heterodera schachtii*: Sindhu AS, Maier TR, Mitchum MG, Hussey RS, Davis EL, Baum TJ: Effective and specific in planta RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success.J Exp Bot 2009, 60:315–324

Transgenic soybean: Klink VP, Kim K-H, Martins V, MacDonald MH, Beard HS, Alkharouf NW, Lee S-K, Park S-C, Matthews BF: A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of development of female Heterodera glycines cyst formation during infection of Glycine max. Planta 2009, 230:53-71

2. Root-knot nematodes

Transgenic *Arabidopsis thaliana* became resistant to *Meloidogyne incognita*: Huang GZ, Allen R, Davis EL, Baum TJ, Hussey RS: Engineering broad rootknot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proc Natl Acad Sci U S A 2006, 103:14302–14306. (arabidopsis)

Transgenic tobacco: Yadav BC, Veluthambi K, Subramaniam K: Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. Mol Biochem Parasitol 2006, 148:219-222

Transgenic soybean: Ibrahim HMM, Alkharouf NW, Meyer SLF, Aly MAM, Gamal El- Din AY, Hussein EHA, Matthews BF: Post-transcriptional gene silencing of root-knot nematode in transformed soybean roots. Exp Parasitol 2011, 127:90-99.

3. Root lesion nematodes

Transgenic walnut became resistant to *Pratylenchus vulnus*: Sriema L Walawage, Monica T Britton, Charles A Leslie, Sandra L Uratsu, YingYue Li and Abhaya M Dandekar (2013) Stacking resistance to crown gall and nematodes in walnut rootstocks. BMC Genomics 14:668

Resistance against insects

1. Chewing insects

Tobacco engineered to have RNAi activity targeting the cytochrome p450 monoxygenase gene (CYPAE14) of the cotton bollworm, *Helicoverpa armigera* inhibited expression of the CYPAE14 in this lepidopteron pest. The inhibition of CYPAE14 expression in these insects led to their increased sensitivity to the natural defence compound, gossypol, produced by the plant.

Mao, Y., Tao, X., Xue, X., Wang, L., Chen, X., 2011. Cotton plants expressing CYP6AE14 double-stranded RNA show enhanced resistance to bollworms. Transgenic Res. 20, 665–673.

Corn plants expressing dsRNA directed against the vacuolar ATPase gene (V-ATPase) of the western corn rootworm (WCR), *Diabrotica virgifera*, effected RNAi activity to this gene in the insect and the transgenic plants exhibited a resistance to feeding damage by this lepidopteron pest.

Baum, J., Bogaert, T., Clinton, W., Heck, G., Feldmann, P., 2007. Control of coleopteran insect pests through RNA interference. Nat. Biotechnol. 25, 1322– 1326.

Resistance against insects

2. Phloem-feeding hemipteran insects

Transgenic rice expressing dsRNA directed against three separate genes expressed in the midgut of the brown plant hopper, *Nilaparvata lugens Stål* showed a decrease in target gene expression in this insect however, no lethal phenotype was detected.

Zha, W., Peng, X., Chen, R., Du, B., Zhu, L., 2011. Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect Nilaparvata lugens. PLoS One 6, e20504.

Transgenic Arabidopsis induced RNAi activity in the gut of the aphid *Myzus persicae* directed toward the Receptor of activated kinase (Rack-1) gene.

Pitino, M., Coleman, A., Maffei, M., Ridout, C., Hogenhout, S., 2011. Silencing of aphid genes by dsRNA feeding from plants. PLoS ONE 6, e25709.

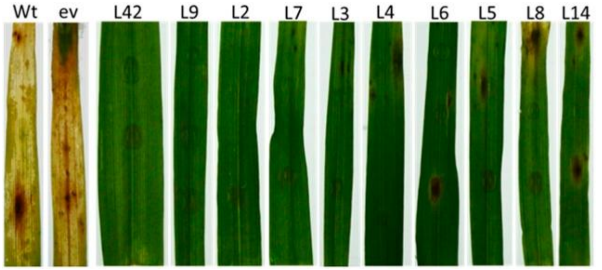
Transgenic tobacco showed enhanced resistance against whitefly.

Nidhi Thakur, Santosh Upadhyay, Praveen Verma, Krishnappa Chandrashekar, Rakesh Tuli, Pradhyumna K. Singh (2014) Enhanced Whitefly Resistance in Transgenic Tobacco Plants Expressing Double Stranded RNA of v-ATPase A Gene. PLOS One Volume 9 | Issue 3 | e87235

Resistance against fungi

Transgenic potato plants, which express RNA interference constructs targeted against plasma membrane-localized SYNTAXIN-RELATED 1 (StSYR1) grew normally and showed increased resistance to *Phytophthora infestans*.
Eschen-Lippold L, Landgraf R, Smolka U, Schulze S, Heilmann M, Heilmann I, Hause G, Rosahl S. (2012) Activation of defense against *Phytophthora infestans* in potato by down-regulation of syntaxin gene expression.

Transgenic banana
Mumbanza, Kiggundu, Tusiime, Tushemereirwe, Niblett and Bailey (2012) In vitro antifungal activity of synthetic dsRNA molecules against two pathogens of banana, *Fusarium oxysporum f. sp. cubense* and *Mycosphaerella fijiensis* Pest Manag Sci **69**: 1155–1162

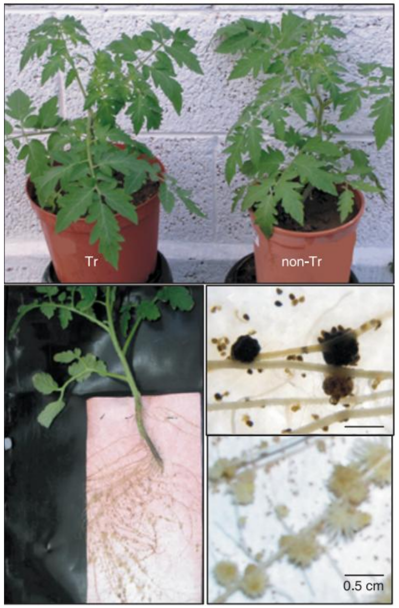


Transgenic Arabidopsis and barley
Aline Koch, Neelendra Kumar, Lennart Weber, Harald Keller, Jafargholi Imani, and Karl-Heinz Kogela, Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase–encoding genes confers strong resistance to *Fusarium* species 19324–19329 | PNAS | November 26, 2013 | vol. 110 | no. 48

Resistance against viruses

Beet Necrotic Yellow Vein Virus (BNYVV)-resistant tobacco
Potato Virus Y (PVY)-resist-ant potato
Papaya Ring Spot Virus type W (PRSV-W)-resistant *Cucumis melo* L. var. *canta-lupensis*
Plum Pox virus (PPV)-resistant *Nicotiana benthamiana* and *Prunus domestica*
Cucumber Green Mottle Mosaic Virus (CGMMV)-resistant *N. benthamiana*
Rice Stripe Virus. Two susceptible rice japonica varieties, Suyunuo and Guanglingxiangjing
African cassava mosaic virus (ACMV) resistant cassava
Cassava Brown Streak Disease (CBSD) in cassava (*M. esculenta*)
Mung-bean Yellow Mosaic India Virus in *Vigna mungo*
geminivirus-resist-ant BGMV-resistant common bean
Tomato Yellow Leaf Curl Virus (TYLC)-resistant tomato
Rice Tungro Bacilliform Virus (RTBV)- resistant rice
Citrus Tristeza Virus (CTV)-resistant mexican lime
Potato Spindle Tuber Viroid (PSTVd) in tomato

Resistance against parasitic weeds



The level of endogenous M6PR mRNA in the tubercles and underground shoots of *Orobanche aegyptiaca* grown on transgenic tomato plants expressing M6PR dsRNA was reduced by 60–80 % with a significant decrease in mannitol level and a significant increase in the percentage of dead *O. aegyptiaca* tubercles.

Aly R, Cholakh H, Joel DM, Leibman D, Steinitz B, Zelcer A, Naglis A, Yarden O, Gal-On A (2009) Gene silencing of mannose 6-phosphate reductase in the parasitic weed *Orobanche aegypti-aca* through the production of homologous dsRNA sequences in the host plant. Plant Biotechnol J 7:487–498

A parasitic weed-resistant variety of maize was developed using RNAi based resistance to *Striga asiatica.*

De Framond A, Rich PJ, McMillan J, Ejeta G (2007) Effects of Striga parasitism of transgenic maize armed with RNAi constructs targeting essential *S. asiatica* genes.
In: Ejeta G, Gressel J Integrating new technologies for striga control.
World Scientific Publishing Co. pp 185–196
Yoder JI, Gunathilake P, Wu B, Tomilova N, Tomilov AA (2009) Engineering host resistance against parasitic weeds with RNA interference. Pest Manage Sci 65:460–466

Metabolism manipulation by RNAi

1. Industrial traits

Simultaneous improvement of fibre quality, early-flowering, early-maturity and productivity in Upland cotton (G. hirsutum) is a challenging task for conventional breeding. The influence of red/far-red light ratio on the fibre length prompted the suppression of the cotton PHYA1 gene. RNAi lines exhibited vigorous root and vegetative growth, early-flowering, significantly improved upper half mean fibre length and an improvement in other major fibre characteristics. These results should aid in the development of early-maturing and productive Upland cultivars with superior fibre quality.

Ibrokhim Y. Abdurakhmonov, Zabardast T. Buriev, Sukumar Saha, Johnie N. Jenkins, Abdusattor Abdukarimov & Alan E. Pepper (2013) Phytochrome RNAi enhances major fibre quality and agronomic traits of the cotton Gossypium hirsutum L. Nature Communications | 5:3062

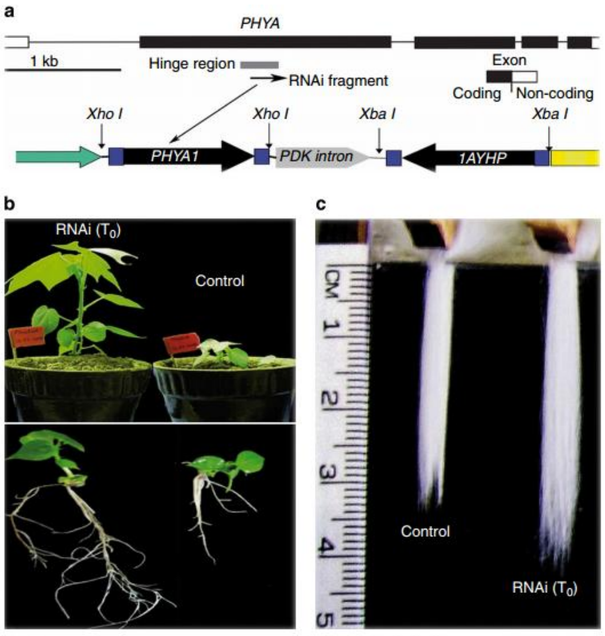
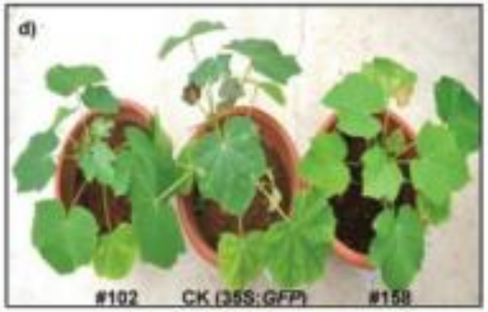


Figure 1 | Effects of *PHYA1* RNAi in cotton. (**a**) Schematic representation of *PHYA* gene, RNAi fragment position and pHellsgate-8::*PHYA1* RNAi plasmid; (**b**) shoot and root development and (**c**) fibre length characteristics of T_0-generation *PHYA1* RNAi and control cotton plants, somatically regenerated by tissue culture. PDK intron—a pyruvate dehydrogenase kinase intron; blue boxes represent *att* sites attached to the target gene fragment.

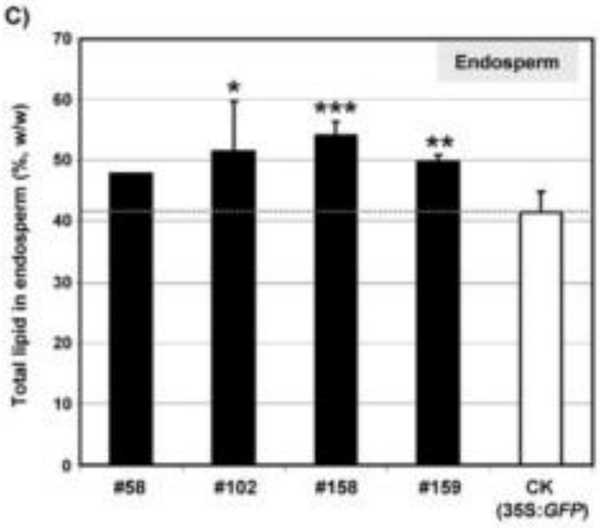
Metabolism manipulation by RNAi

1. Industrial traits

Arabidopsis mutants deficient in SDP1 accumulate high levels of oils, probably due to blockage in TAG degradation. This knowledge was applied to engineer increased seed oil content in the biodiesel plant Jatropha curcas. Jatropha JcSDP1-RNAi plants accumulated 13 to 30% higher total seed storage lipid.



Mi Jung Kim, Seong Wook Yang, Hui-Zhu Mao, Sivaramakrishnan P Veena, Jun-Lin Yin and Nam-Hai Chua (2014) Gene silencing of Sugar-dependent 1 (JcSDP1), encoding a patatin-domain triacylglycerol lipase, enhances seed oil accumulation in Jatropha curcas. Biotechnology for Biofuels 2014, 7:36



Metabolism manipulation by RNAi

1. Industrial traits

Lignin is a major cell wall component of vascular plants that provides mechanical strength and hydrophobicity to vascular vessels. However, the presence of lignin limits the effective use of crop straw in many agroindustrial processes. Transgenic maize plants were generated in which the expression of a lignin biosynthetic gene encoding CCoAOMT, a key enzyme involved in the lignin biosynthesis pathway was downregulated by RNAi. These transgenic plants exhibited a 22.4% decrease in lignin content and a 23.3% increase in cellulose content compared with WT plants.

Xiaoyu Li, Wenjuan Chen, Yang Zhao, Yan Xiang, Haiyang Jiang, Suwen Zhu and Beijiu Cheng (2013) Downregulation of caffeoyl-CoA O methyltransferase (CCoAOMT) by RNA interference leads to reduced lignin production in maize straw. Genetics and Molecular Biology, 36, 4, 540-546 (2013)

Figure 1 - Phenotype of RNAi *CCoAOMT* transgenic pla plants grown in soil.

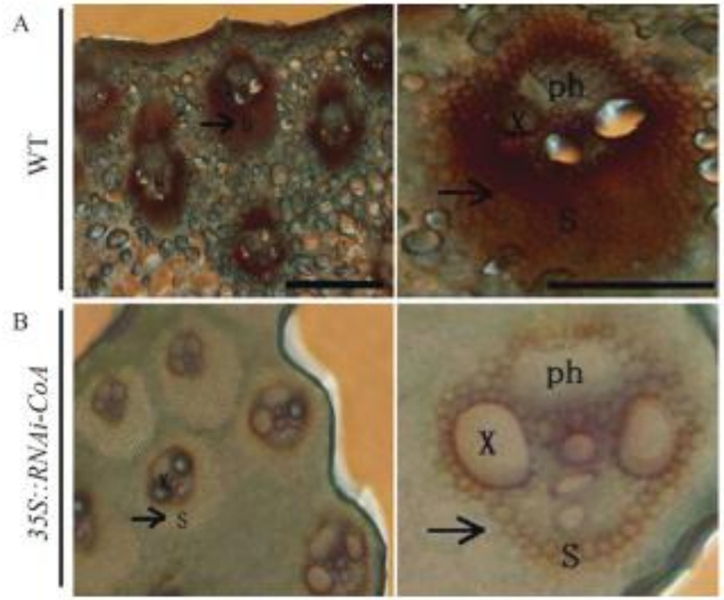


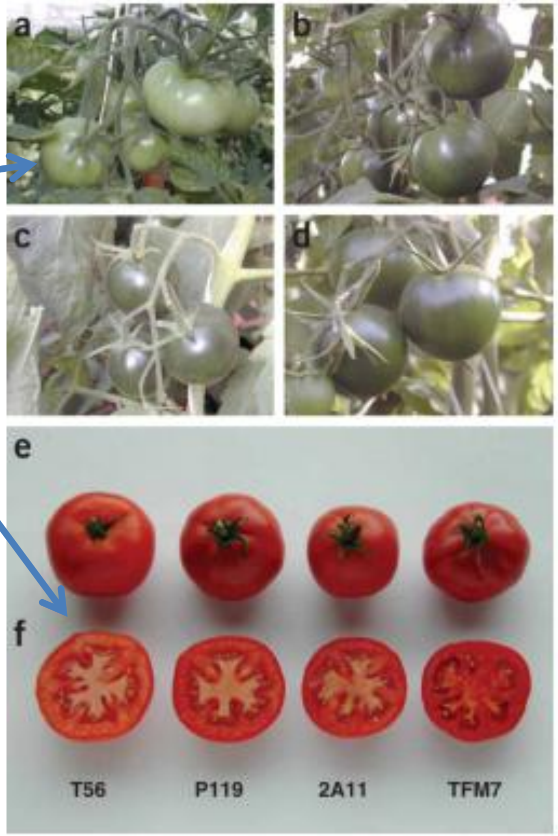
Figure 5 - Histological staining for lignin deposition in stem cross-sections of WT (A) and RNAi *CCoAOMT* transgenic maize plants using Wiesner reagent (B). *X* xylem, *S* sclerenchyma, *ph* phloem.

Metabolism manipulation by RNAi

2. Nutritional improvement

RNAi-mediated suppression of an endogenous photomorphogenesis regulatory gene, DET1, using fruit-specific promoters significantly increased carotenoid and flavonoid in the fruit without affecting the level of other components. This was the first report of simultaneous elevation of flux through two independent health-related biosynthetic pathways with no negative collateral effects on fruit yield or quality.

wild type



3 different promoters

Davuluri, G. R., Tuinen, A., & Fraser, P. D. (2005). Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. Nature Biotechnology, 23(7), 890–895.

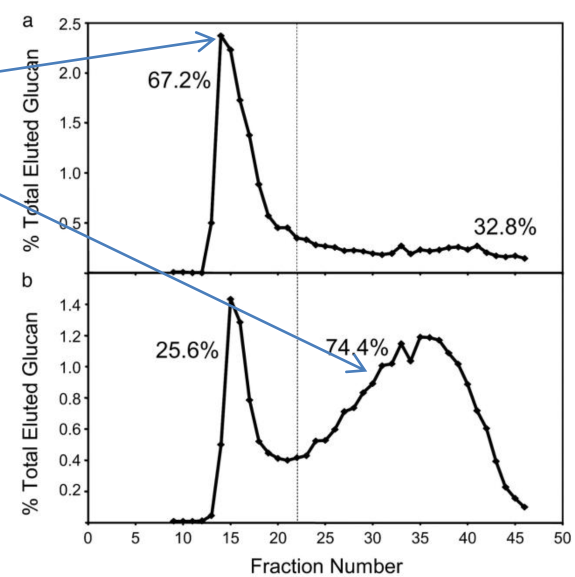
Metabolism manipulation by RNAi

2. Nutritional improvement

The major plant-derived carbohydrate is starch, which is composed of amylopectin and amylose polysaccharides. The high calorie food is not always desirable as excess of digested calories are associated with obesity and other diseases in developed countries. In cooked foods, amylose molecules form digestion-resistant complexes that are part of healthy dietary fiber . RNA interference has been used to downregulate the two different isoforms of starch-branching enzyme (SBE) II (SBEIIa and SBEIIb) in wheat endosperm to raise its amylose content. The suppression of both SBEIIa and SBEIIb expression resulted in starch containing more than 70 % amylose content. Feeding this high amylose wheat grain to rats in a diet as a whole meal resulted improvement in bowel function as compared to standard wholemeal wheat. The results of this study revealed the significant potential of this high amylose wheat to improve human health through its resistant starch content.

amylose

amylopectin



RNAi line

wt

Regina A, Bird A, Topping D, Bowden S, Freeman J, Barsby T, Kosar-Hashemi B, Li Z, Rahman S, Morell M. (2006) High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proc Natl Acad Sci U S A. 2006 Mar 7;103(10):3546-51.

Metabolism manipulation by RNAi

2. Nutritional improvement

It has been shown that starch phosphorylation and dephosphorylation are critical components leaf starch degradation. Glucan water dikinase (GWD) adds phosphate to starch, and phosphoglucan phosphatase (SEX4) removes these phosphates. Elevated leaf starch content in maize leaves was engineered by making an RNAi construct against a gene in maize that appeared to be homologous to AtGWD. Leaf starch content at the end of a night period in engineered maize plants was 20-fold higher than in untransformed plants with no impact on total plant biomass.

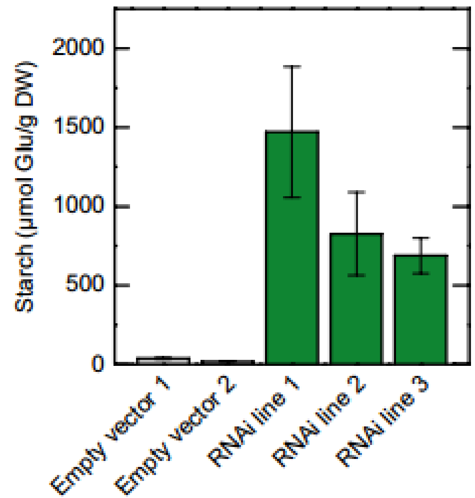


Figure 6 Starch levels in transgenic maize RNAi lines against the GWD like gene. Values are mean ± SE (n = 5). GWD, glucan, water dikinase.

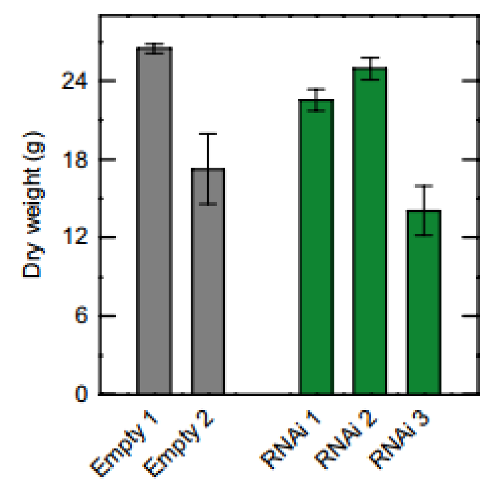


Figure 9 Total above ground biomass of transgenic maize lines.

Weise SE, Aung K, Jarou ZJ, Mehrshahi P, Li Z, Hardy AC, Carr DJ, Sharkey TD (2012) Engineering starch accumulation by manip-ulation of phosphate metabolism of starch. Plant Biotechnol J. Volume 10, Issue 5, pages 545–554,

Metabolism manipulation by RNAi

2. Nutritional improvement

Celiac disease (CD) is an enteropathy triggered by the ingestion of gluten proteins from wheat and similar proteins from barley and rye. The only available treatment for the disease is a lifelong gluten-exclusion diet. RNAi was applied to down-regulate the expression of gliadins in bread wheat. The expression of gliadins was strongly down-regulated in the transgenic lines. The total gluten extracts were unable to elicit T-cell responses for three of the transgenic wheat lines. This work shows that the down-regulation of gliadins by RNAi can be used to obtain wheat lines with very low levels of toxicity for CD patients.

Gil-Humanes J, Pisto´n F, Tollefsen S, Sollid LM, Barro F (2010) Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. Proc Natl Acad Sci U S A 107: 17023–17028.

Metabolism manipulation by RNAi

2. Nutritional improvement

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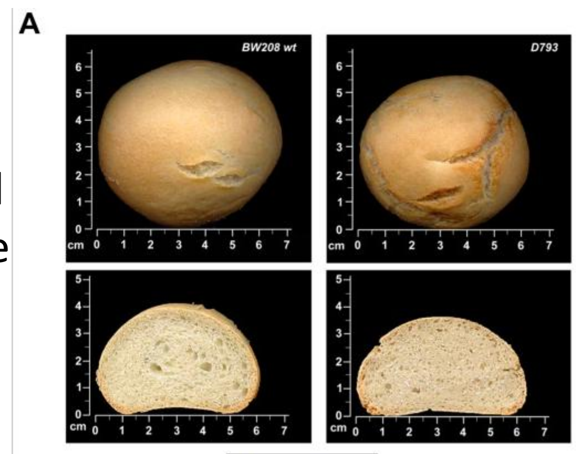
Gil-Humanes, Piston, Altamirano-Fortoul, Real, Comino, Sousa, M. Rosell, Barro (2014) Reduced-Gliadin Wheat Bread: An Alternative to the Gluten-Free Diet for Consumers Suffering Gluten-Related Pathologies. PLOS One Volume 9 | Issue 3 | e90898

wild type



RNAi

Metabolism manipulation by RNAi

2. Nutritional improvement

The consumption of alpha-linolenic acid (18:3) was found to be unhealthy for human as well as animals. The reduction of alpha-linolenic acid (18:3) is good to improve soybean oil flavour and stability with reduced need for its hydrogenation. The linoleic acid (18:2) is converted into alpha-linolenic acid (18:3) in presence of enzyme omega-3 fatty acid desaturase. RNAi was used to down regulation of omega-3 fatty acid desaturase (*Gm*FAD3A, *Gm*FAD3B and *Gm*FAD3C) and transgenic soybean seed has been reported to have 1–3 % of alpha-linoleic acid in comparison with 7–10 % in non-transgenic soybean seed.

Flores T, Karpova O, Su X, Zeng P, Bilyeu K, Sleper DA, Nguyen HT, Zhang ZJ (2008) Silencing of GmFAD3 gene by siRNA leads to low alpha-linolenic acids (18:3) of fad3-mutant phenotype in soybean [Glycine max (Merr.)]. Transgenic Res 17:839–850

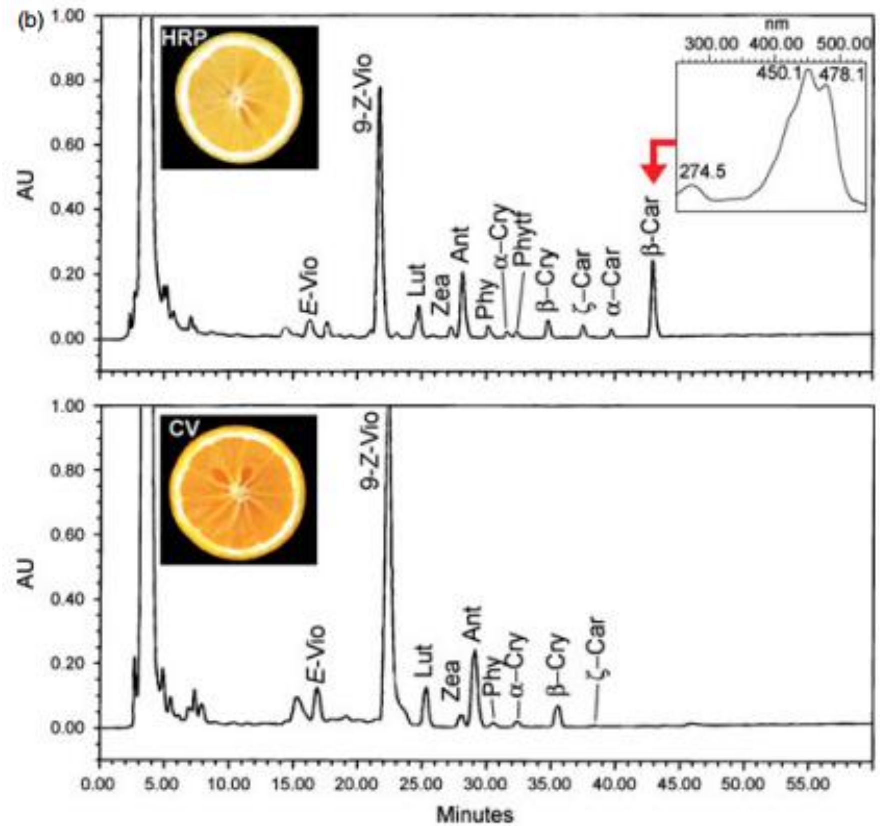
Metabolism manipulation by RNAi

2. Nutritional improvement

Orange is a major crop and an important source of health-promoting bioactive compounds. Increasing the levels of specific antioxidants in orange fruit through metabolic engineering could strengthen the fruit's health benefits. b-carotene content of orange fruit was enhanced through blocking by RNAi the expression of an endogenous b-carotene hydroxylase gene (Csb-CHX) that is involved in the conversion of b-carotene into xanthophylls.

RNAi

WT



Elsa Pons, Berta Alquezar, Ana Rodrıguez, Patricia Martorell, Salvador Genoves, Daniel Ramon,Marıa Jesus Rodrigo, Lorenzo Zacarıas and Leandro Pena (2014) Metabolic engineering of b-carotene in orange fruit increases its in vivo antioxidant properties. Plant Biotechnology Journal (2014) 12, pp. 17–27

Metabolism manipulation by RNAi

2. Nutritional improvement

RNAi has been successfully used to generate a dominant high-lysine maize variant by knocking out the expression of the 22-kD maize zein storage protein, a protein that is poor in lysine content. Traditional breeding has been successful only for the screening of a recessive lysine-rich mutant called opaque 2 (O2). The O2 gene encodes a maize basic leucine zipper transcriptional factor that controls the expression of a subset of storage proteins, including the 22-kDa zein storage protein. Although it is rich in lysine, the opaque 2 mutant is not very useful in agriculture because of its adverse effects on seed quality and yield. By contrast, downregulation of the maize lysine-poor 22-kDa zein gene via RNAi does not alter the general functions of O2, but generates quality and normal maize seeds with high levels of lysine-rich proteins.

Segal, G. et al. (2003) A new opaque variant of maize by a single dominant RNA-interference-inducing transgene. Genetics 165, 387–397

Metabolism manipulation by RNAi

2. Nutritional improvement – allergen elimination

major apple allergen, Mal d1 - Gilisen et al. (2005) J Allergy Clin Immunol 115:364–369

25 % reduction of Ara h 2 content in peanut extract – Dodo et al (2008) Plant Biotech-nol J 6:135–145

Lyc e3 in tomato - Le et al. (2006) Plant Biotechnol J 4:231–242

linamarin content, a cyanogenic substance in cassava by suppression of cytochrome P450 - Siritungam and Sayre (2003) Planta 217:367–373 and Jørgensen et al. (2005) Plant Phys-iol 139:363–374

LFS in onion (tearless onion) - Eady et al. (2008) Plant Physiol 147:2096–2106

beta-*N*-oxalyl-ami-noala-nine-l-alanine (BOAA) in grass pea (*Lathyrus sativus*), which is consumed in India, Bangladesh, Ethiopia, etc. - Angaji et al. 2010. Plant Omics J 3:77–84