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Review Paper

RNA interference in fruit and vegetables for crop improvement

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ABSTRACT

RNA interference is a very efficient knockdown technology in plants as it is useful for genetic improvement, in plants with low transformation efficiencies. Down regulation of a particular gene can be achieved by mutation-based reverse genetics, but its use is more limited than that of RNAi. Although the basic concept of the application of transgene-based RNAi for genetic improvement of crop plants has been established, further feasibility studies are needed for its wider application. One of the major purposes of the present review article is to help policy makers in food deficient countries to understand how scientific breakthroughs such as RNAi technology may be helpful in tackling this gigantic problem of feeding an additional 2 billion people over the next 30 years from an increasingly fragile natural resource base. However, any new technology involving the gene manipulation may be opposed by anti-GM groups severely limiting its effectiveness or wider use. Since this technology offers a great potential in understanding gene functions and utilize them to improve crop quality and production, it is a matter of time before we see the products of this RNAi research in the farmers' fields around the world.

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RNA interference (RNAi) is a post-transcriptional gene-silencing phenomenon induced by double-stranded RNA. It has been widely used as a knock down technology to analyze gene function in various organisms. Although RNAi was first discovered in worms, related phenomena such as posttranscriptional gene silencing and coat protein mediated protection from viral infection had been observed in plants prior to this. The technology became a powerful tool to understand the function of individual genes also proved useful for molecular breeders to produce improved crop varieties. Introduction of a piece of double stranded RNA (dsRNA) into the cytosol initiates the phenomenon of RNAi, in turn activating a pathway culminating in the degradation of the targeted gene transcript (Agrawal *et al.,* 2003). In addition to RNA degradation upon activation of the RNAi pathway, there are also cases where the promoter region of the gene is silenced through methylation (Mette *et al.,* 2000). In plants, RNAi is often achieved through transgenes that produce hairpin RNA. For genetic improvement of crop plants, RNAi has advantages over antisense-mediated gene silencing and co-suppression, in terms of its efficiency and stability. It also offers advantages over mutation-based reverse genetics in its ability to suppress transgene expression in multigene families in a regulated manner. More recently, research in this field has been directed to several other areas including microRNA's (Bartel, 2004; Pasquinelli, 2002), promoter methylation (Matzke *et al.,* 2004), and hairpin RNA (Wesley *et al.,* 2001). Simultaneously, results obtained in these areas have found practical applications in crop improvements such as in the production of potato virus Y (PVY) resistant potatoes (Smith *et al*., 2000). Other important areas of RNAi will also be discussed with special reference to its applicability for production of improved varieties of cereal, fruit, cash and vegetable crops.

A surprising observation was made in petunias. While trying to deepen the purple color of these flowers, Rich Jorgensen and colleagues introduced a pigment-producing gene under the control of a powerful promoter. Instead of the expected deep purple color, many of the flowers appeared variegated or even white. Because of this observation the phenomenon was first named "co-suppression of gene expression" but the molecular

Table 1: Chronological history of early RNAi and RNAi related phenomenon

Year	Plants	Animals
1986	Coat protein mediated protection	
1990	Co-suppression	
1993		Micro RNA (small temporal RNA)
1995	Virus-induced gene silencing	The first description of RNAi
1998	hpRNA transgene	RNAi
1999	siRNA, RdRP	*In vitro* RNAi
2000		RNA-induced silencing complex
2001		Dicer

Makoto Kubaba (2004)

mechanism remained unknown. Different chronological history given in Table 1.

RNA inference in fruits:

Banana:

Rodoni *et al.* (1999) developed banana varieties resistant to the Banana Bract Mosaic Virus (BBrMV) by carefully designing an RNAi vector aimed at silencing the coat protein (CP) region of the virus, scientists may be able to develop a banana variety that is resistant to BBrMV and yet safe to eat. The CP region of the different strains of virus is highly conserved and as such silencing of this gene in other varieties of banana will not pose a problem.

Strawberry:

Hoffman *et al.* (2006) developed Intron-containing constructs encoding self-complementary 'hairpin' RNA (ihpRNA) have the potential to efficiently silence genes in a range of plant species. The silencing of a ripening-related chalcone synthase (CHS) gene in strawberry fruits (Fragaria x ananassa cv. Elsanta) by a construct (ihpRNA) containing the partial sense and corresponding antisense sequences of CHS separated by an intron obtained from a F. x ananassa quinone oxidoreductase gene. An Agrobacterium strain carrying a T-DNA expressing the ihpRNA transgene was injected with a syringe into the receptacles of growing fruits still attached to the plant about 14 days after pollination. As a consequence of the reduced levels of CHS mRNA and enzymatic CHS activity, the levels of anthocyanins were downregulated and precursors of the flavonoid pathway were shunted to the phenylpropanoid pathway leading to large increases in levels of (hydroxy) cinnamoyl glucose esters. They concluded that this technique in combination with metabolite profiling analysis will be useful for studying the function of unknown genes during the development and ripening of strawberry fruit.

Apple:

Cao *et al.* (2004) obtained antisense and sense gene fragments (710 base pairs) of apple polyphenol oxidase (APPO) gene by RT-PCR amplification, using the total RNAs isolated from ripen apple fruit as the template. These two fragments were ligated with a 1000 bp spacer, YYT (crtW+crtY fusion) gene, which is relative to carotenoid synthesization in subcocci. The full-length 2446 bp-target gene was then inserted into plant binary vector pYPX145 to generate the recombinant plasmid pYF7704, which carried the expression unit, of APPO dsRNA. pYF7704 was transformed to apple (Malus x domestica) var. Red Fuji via *Agrobacterium tumefaciens* mediated leaf disc transformation. With the selection of Karamycin and GUS detection assays, transgenic shoots of A PPO dsRNA were obtained. The results of FQ-RT-PCR indicated that APPO mRNA level was suppressed to 91.69% in transgenic shoots compared to wide shoots. The data suggested that dsRNAi technology on apple polyphenol oxidase is feasible to be utilized in transgenic shoots.

RNA interference in vegetable:

Michael *et al.* (1997) studied the resistance to tobacco etch potyvirus (TEV) in *Capsicum annuum* cv. Dempsey is conferred by the recessive gene *et"*. When Dempsey pepper plants were inoculated with either of two TEV isolates, virus did not accumulate in inoculated or noninoculated leaves. In contrast, each TEV isolate replicated to high levels in *C. annuum* cv. Jupiter, a susceptible parent of Dempsey pepper. To determine the basis of the resistance, Dempsey and Jupiter pepper plants were inoculated with a TEV isolate (TEV-HAT) engineered to express the b-glucuronidase gene (TEV-GUS). TEV-GUS moved cell-to-cell and systemically infected Jupiter pepper plants. In contrast, there was no evidence of any single-cell infection foci induced by TEV-GUS in Dempsey pepper plants. Northern blot analysis of protoplasts inoculated with TEV-HAT RNA indicated that

virus RNA did not accumulate in Dempsey pepper protoplasts, whereas TEV-HAT did replicate and accumulate in protoplasts from Jupiter pepper plants. Therefore, concluded that, the resistance to TEV conferred by the *et a* gene is due to interference with virus RNA accumulation.

***Lathyrus sativus* (leafy vegetable):**

In Ethiopia, Bangladesh and India, the people in the lower socioeconomic class use a leafy vegetable known as *Lathyrus sativus*. It is a leguminous crop and contains a neurotoxin called β-oxalylaminoalanine-L-alanine (BOAA) (Spencer *et al*., 1986). People consuming this vegetable suffer from a paralytic disease called, lathyrism. The disease paralyses people both temporarily and permanently, however, the effects can be somewhat reduced if the plant is boiled prior to consumption. Paralysis in the limbs is a known symptom of BOAA, yet people still consume this vegetable in times of famine. This species is remarkably suited to grow in marginal and inhospitable land without irrigation, fertilizer, and pesticides. It flourishes also times of devastating flood and drought, when no other food crop survives. This is an instance where RNAi technology can be used to silence the gene(s) responsible for production of BOAA. There may be one difficulty; in that the BOAA genes may be linked to genes, which confer immunity to this unique crop or impart drought and flood tolerance. Bringing down the levels of BOAA to a safe concentration, rather than totally silencing the concerned genes, may overcome this obstacle.

Potato:

Kim (2008) developed patatin knockdown potato tubers using RNA interference (RNAi) technology, for the production of human-therapeutic glycoproteins. patatin encoded by a multi-gene family are one of the major storage glycoproteins in potato tubers. Potato tubers have recently emerged as bioreactors for the production of human therapeutic glycoproteins (vaccines). Increasing the yield of recombinant proteins, targeting the produced proteins to specific cellular compartments, and diminishing expensive protein purification steps are important research goals in plant biotechnology. The potato patatins were eliminated almost completely via RNA interference (RNAi) technology to develop potato tubers as a more efficient protein expression system.

Sweet potato:

Takiko *et al.* (2006) increased amylase content in the storage roots of sweetpotato (*Ipomoea batatas* (L.) Lam. cv. Kokei 14) by RNA interference of starch branching enzyme II gene (IbSBEII). 10 to 20% of the starch is essentially unbranched linear amylose and the other major component is branched amylopectin. The starch branching enzymes, which are responsible for production of amylopectin to form a -1,6-linkages in the glucan can be divided into two classes, class A (e.g. potato and maize SBEII, pea SBEI) and class B (e.g. potato and maize SBEI, pea SBEII). On the bases of the registered cDNA of sweetpotato SBEII (*IbSBEII*) encoding class A branching enzyme, we constructed doublestranded RNA (dsRNA) interference vectors and introduced them into sweetpotato genome via *Agrobacterium*-mediated gene transformation. We obtained eight independent transgenic plants by using two kinds of RNA interference (RNAi) constructs, encoding *GBSSI* 1st intron-spliced RNA or a GUS fragment-spliced RNA, respectively. All transgenic plants were confirmed not to express *IbSBEII* by RT-PCR and to have the starch with a higher amylose content than the nontransgenic control (up to 25% compared to 10% in the control). Both constructs induced the same level of silencing of *IbSBEII* in all transgenic plants. The morphological characters showed no significant differences between the transgenic and control plants. Starch yield of transgenic tubers was slightly lower than that of non-transgenic tubers. The starch granules of the transgenic plants were similar to those of typical sweetpotato starches in shape and the distribution in granule size, but slightly different in grain structure.

Each starch solution they were stained with iodine solution. Fig. 1 shows the stained solution of starch from three transgenic lines compared with that from the non transgenic line and amylose-free starch. Starch solutions from the transgenic lines were stained to a dark blue colour than that from non-transgenic line, suggesting that the amylose contents in transgenic starch were high. When the amylose content was determined by the blue value

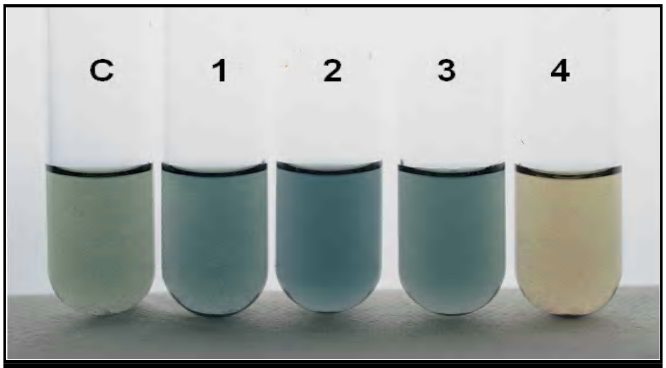

Fig. 1: Iodine staining pattern of starch solution from nontransgenic Kokei 14 (c), transgenic lines with dsRNA of *IbSBEII* (1, ASIS-1; 2, ASGS 1; 3, GS-2) and a transgenic line with dsRNA of *GBSSI* (4)

Table 2 : The yield and amylose content of starches from transgenic sweet potato roots

Sample	Total fresh weight (g)	Starch yield (g)	Amylose content (%)	Control/transgenic
Control	281.7	5.8	10.25	100
ASGS-1	259.6	5.2	17.90	175
ASGS-2	181.8	4.7	23.33	228
ASGS-3	541.3	4.1	15.67	153
ASGS-4	144.5	5.9	24.23	236
ASIS-1	277.0	4.5	20.00	195
ASIS-2	244.4	4.9	23.35	228
ASIS-3	173.5	4.4	24.35	242
ASIS-5	205.2	4.2	23.85	233

Takiko *et al.* (2005)

1) Control, non-transgenic plants; ASGS-1_-4, transgenic plants with pIbSBEII SAGA; ASIS-1_-5, transgenic plants with pIbSBEII-ASIA.

absorbance at 680 nm, starches from the transgenic tubers contained 15.4% to 24.3% of amylose, while nontransgenic starch contained only 10% (Table 2). Starches of transgenic lines contained amylose two-fold as much as the control.

Tomato:

Elio *et al.* (2007) studied the formation of seedless fruits in the absence of functional fertilization, is a desirable trait for several important crop plants, including tomato (*Solanum lycopersicum*). Seedless fruits can be of great value for consumers, the processing industry, and breeding companies. RNAi is novel strategy to obtain parthenocarpic tomatoes by down-regulation of the flavonoid biosynthesis pathway using RNA interference (RNAi)-mediated suppression of chalcone synthase (CHS), the first gene in the flavonoid pathway. In CHS RNAi plants, total flavonoid levels, transcript levels of both *Chs1* and *Chs2*, as well as CHS enzyme activity were reduced by up to a few per cent of the corresponding wild-type values. Surprisingly, all strong *Chs*-silenced tomato lines developed parthenocarpic fruits. Although a relation between flavonoids and parthenocarpic fruit development has never been described, it is well known that flavonoids are essential for pollen development and pollen tube growth and, hence, play an essential role in plant reproduction. The observed parthenocarpic fruit development appeared to be pollination dependent, and *Chs* RNAi fruits displayed impaired pollen tube growth.

Matthew *et al.* (2004) developed the FLAVR SAVR tomato through the use of antisense RNA to regulate the expression of the enzyme polygalacturonase (PG) in ripening tomato fruit. This enzyme is one of the most abundant proteins in ripe tomato fruit and has long been thought to be responsible for softening in ripe tomatoes. The FLAVR SAVR tomato is the first genetically engineered whole food to be sold in commerce.

Limitation:

The initial limitation of RNAi technology is designing an effective siRNA sequence. Advancements in siRNA delivery (such as the enzymatic synthesis of siRNAs from T7 promoters) have placed constraints on which sequences of the target genes can even be considered for use. Many of these constraints depend on the type of polymerase ultimately used to recognize and amplify the siRNA sequence. However, even following the recommended rules for siRNA design does not ensure effective silencing of the target gene. The efficacy of siRNA-mediated suppression of gene expression depends on a number of factors, including not only the chosen siRNA sequence but also the structure of the siRNA, and the receptiveness of the cell type to siRNA uptake. In addition, the half life of the target message and/or protein needs to be considered in order to achieve optimal silencing.

The use of RNAi for therapeutic purposes will depend on other factors as well. Although siRNAs are relatively stable in cell culture conditions, they require enhanced nuclease and thermodynamic stability when in circulation *in vivo*. Chemical modifications of siRNAs to enhance this stability are being explored. While there are mixed opinions as to which type of modification will be most effective at enhancing stability without compromising target silencing activity, advances are being made toward the goal of making siRNAs suitable for therapeutic purposes.

Future prospect:

– To exploit plant-specific gene silencing mechanisms to develop new strategies for the prevention of transgene silencing and for the controlled use of gene silencing for the inactivation of endogenous genes and gene families in various crop plants.

– To combine novel basic research elements with the application of these results in modern agriculture for

the benefit of the consumers.

– To develop strategies for a safe and reliable use of a commercially highly important technology.

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