ACCELERATED PUBLICATION

RNase P as a tool for disruption of gene expression in maize cells

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RNase P, a ribonucleoprotein responsible for the 5′ maturation of precursor tRNAs (ptRNAs) in all organisms, can be enticed to cleave any target mRNA that forms a ptRNA-like structure and sequence-specific complex when bound to an RNA, termed the EGS (external guide sequence). In the present study, F3H (flavanone 3-hydroxylase), a key enzyme in the flavonoid biosynthetic pathway that participates in the formation of red-coloured anthocyanins, was used as a target for RNase P-mediated gene disruption in maize cells. Transient expression of an EGS complementary to the F3H mRNA resulted in suppression of F3H to 29 % of the control, as indicated by a reduced number of anthocyanin-accumulating cells. This decrease was not observed in experiments where a disabled mutant EGS was expressed. Our results demonstrate the potential of employing plant RNase P, in the presence of an appropriate gene-specific EGS, as a tool for targeted degradation of mRNAs.

Key words: external guide sequence, flavanone 3-hydroxylase, maize (*Zea mays*), RNase P.

INTRODUCTION

The recent spate of complete genome sequences has provided new impetus for the development of reliable tools for elucidating gene–function correlations. Frequently, this objective has been accomplished through the employment of loss-of-function genetic approaches. These gene knockdown methodologies include antisense-mediated suppression, ribozymes and RNAi (RNA interference) [1–4]. The absence of data for the temporal and spatial effects on the functioning of currently employed gene silencing strategies, including RNAi, leaves unanswered the question of universal applicability of any one tool and lends support to the development of new and complementary approaches. One promising strategy involves the recruitment of RNase P, an endogenous enzyme in all organisms, to specifically degrade a target mRNA [5,6].

RNase P is a ubiquitous ribonucleoprotein responsible for the 5′ maturation of precursor tRNAs (ptRNAs) in prokaryotes and eukaryotes (Figure 1A; [7–9]). The observation that RNase P can cleave a bipartite RNA complex that structurally resembles a ptRNA led to the idea that any target mRNA that can form a ptRNA-like structure and a sequence-specific complex with an EGS (external guide sequence) RNA can potentially become a non-natural substrate for RNase P cleavage (Figure 1C; [5,6,10]). This idea has been demonstrated successfully in mammalian cell cultures [11–13]. As part of our studies to test the viability of this approach in plants, we reported previously that partially purified rice and maize (*Zea mays*) RNase P, like their mammalian counterparts, are capable of cleaving a non-natural bipartite substrate *in vitro* [14]. In the present study, we have extended our earlier studies by demonstrating successfully the efficacy of RNase P as a tool for disrupting expression of an endogenous gene in maize calli.

MATERIALS AND METHODS

Selection of target site

The region of the F3H (flavanone 3-hydroxylase) cDNA encoding the first 115 amino acid residues [15] was cloned into pUC19 under the control of a T7 RNA polymerase promoter. The *in vitro* transcript made from this partial *F3H* gene was used for RNase T1 enzymic probing to determine the single-stranded regions accessible for EGS binding [16]. These unpaired regions were also validated using the RNA secondary structure predictions (http://www.bioinfo.rpi.edu/applications/mfold). Together, these results helped us identify a favourable site for targeting EGSs (Figure 1B).

Cloning of various constructs used in this study

The wheat (*Triticum* sp.) *U3 snRNA* (small nuclear RNA) promoter, used to drive the expression of EGSs, was obtained by PCR-based amplification from wheat genomic DNA using the Ta-U3-F and Ta-U3-R primers (Table 1). The PCR product was then digested with *Eco*RI and *Bam*HI before cloning into pUC19 to yield pUC19-TaU3Pro.

The WT EGS^{F3H} RNA (WT is wild-type) was designed to include the regions of a typical ptRNA substrate in addition to 11 bp complementary to the target F3H mRNA (Figure 1C). The ΔT EGS^{F3H} RNA differs from its WT counterpart in that the T stemloop equivalent has been deleted (Figure 1D). The WT-EGS^{F3H}-F and WT-EGS^{F3H}-R, and ΔT-EGS^{F3H}-F and ΔT-EGS^{F3H}-R primer pairs (Table 1) were used to construct the *WT-EGS*^{F3H} and *ΔT-EGS*^{F3H} genes respectively. The respective primer pairs were annealed, filled-in using *Taq* DNA polymerase, digested with *Bam*HI and *Hin*dIII, and cloned into pUC19-TaU3Pro downstream of the *U3 snRNA* promoter.

Abbreviations used: BMS, Black Mexican Sweet; DFR, dihydroflavanol reductase; F3H, flavanone 3-hydroxylase; GUS, β-glucuronidase; LUC, luciferase; pol III, RNA polymerase III; ptRNA, precursor tRNA; RNAi, RNA interference; snRNA, small nuclear RNA; WT, wild-type.

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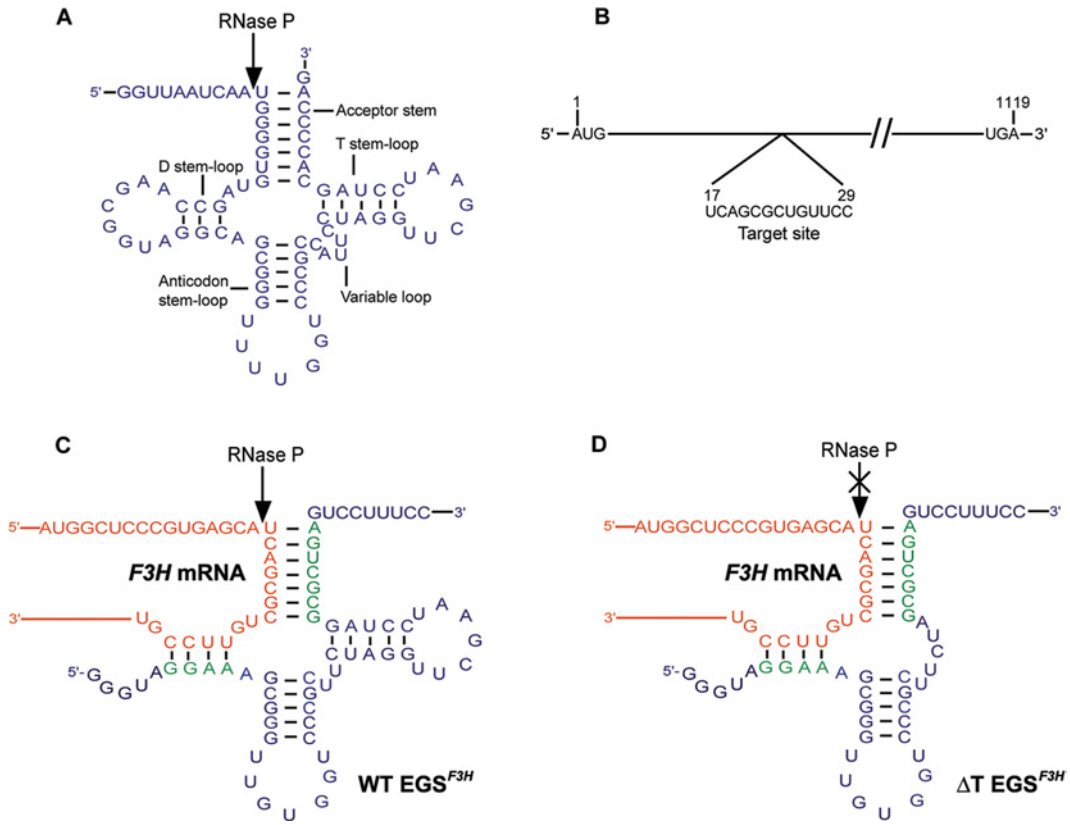

Figure 1 RNase P-mediated cleavage of F3H mRNA

(**A**) The structure of a ptRNA^{Gln}, a typical substrate for RNase P. The arrow indicates the site of cleavage by RNase P. (**B**) Map of the F3H mRNA showing the EGS target site (not drawn to scale). (**C**) A ptRNA-like structure formed by the target mRNA (red) and WT EGS^{F3H}, resulting in a non-natural substrate for RNase P cleavage. In addition to possessing complementary regions to the F3H mRNA (green), the other regions of a typical ptRNA substrate are also present (blue). (**D**) The mutant ΔT EGS^{F3H}, which lacks the T stem-loop-equivalent domain, is not expected to promote RNase P cleavage of the target mRNA.

Table 1 Primers used in the present study

Name	Sequence
Ta-U3-F	5′-GGAATTCGAGACTGATGTCTCTCAGATGG-3′
Ta-U3-R	5′-CGGGATCCGCTTCTTGGTGCCGCGCC-3′
ΔT-EGS^{F3H}-F	5′-CGGGATCCGGGTAGGAAAGCGGGTTGTGGTCCCGCTTCTAGC-3′
ΔT-EGS^{F3H}-R	5′-GGGAAGCTTAAAAAAAAAGAAAAAAGGAAAGGACTCAGCGCTAGAAGCGGGACC-3′
WT-EGS^{F3H}-F	5′-CGGGATCCGGGTAGGAAAGCGGGTTGTGGTCCCGCTTCTAGG-3′
WT-EGS^{F3H}-R	5′-GGGAAGCTTAAAAAAAAAGAAAAAAGGAAAGGACTCAGCGCCTAGGATTCGAACCTAGAAGCGGGACC-3′

The other plasmids used in this study (Figure 2) have been described previously [17].

Transformation

Maize cv. Black Mexican Sweet (BMS) cells were maintained as suspension cultures in Murashige and Skoog medium (Caisson Laboratories) and grown in the dark at 27 °C on a rotary shaker at 150 rev./min. The cells were pre-treated for 12–18 h in liquid medium containing 3 % (w/v) poly(ethylene glycol) 8000 and transformed by microprojectile bombardment with 0.1 μg of *35S::C1 + 35S::R*, 3 μg of *UBI::GUS*, 3 μg of *A1::LUC* and various amounts (1–12 μg) of either WT EGS^{F3H} or ΔT EGS^{F3H} plasmids. The total amount of plasmid DNA in each bombardment was kept constant (18 μg) by supplementing the bombardment cocktail with an appropriate amount of an unrelated plasmid whenever the total amount was <18 μg. An outline of the experimental design is provided in Figure 2(A). The complete protocol for microprojectile bombardment has been described elsewhere [17]. The number of red cells observed in individual transformations with *35S::C1 + 35S::R* averaged 100–200 and ensured robust statistical analysis of phenotypic changes.

RESULTS AND DISCUSSION

Rationale for selection of target gene

The F3H mRNA was chosen as the target for RNase P-mediated cleavage as it encodes a central enzyme necessary for the formation of both flavonols and anthocyanins in the flavonoid biosynthetic pathway (Scheme 1). While flavonols are required for male fertility in plants, anthocyanins are non-essential

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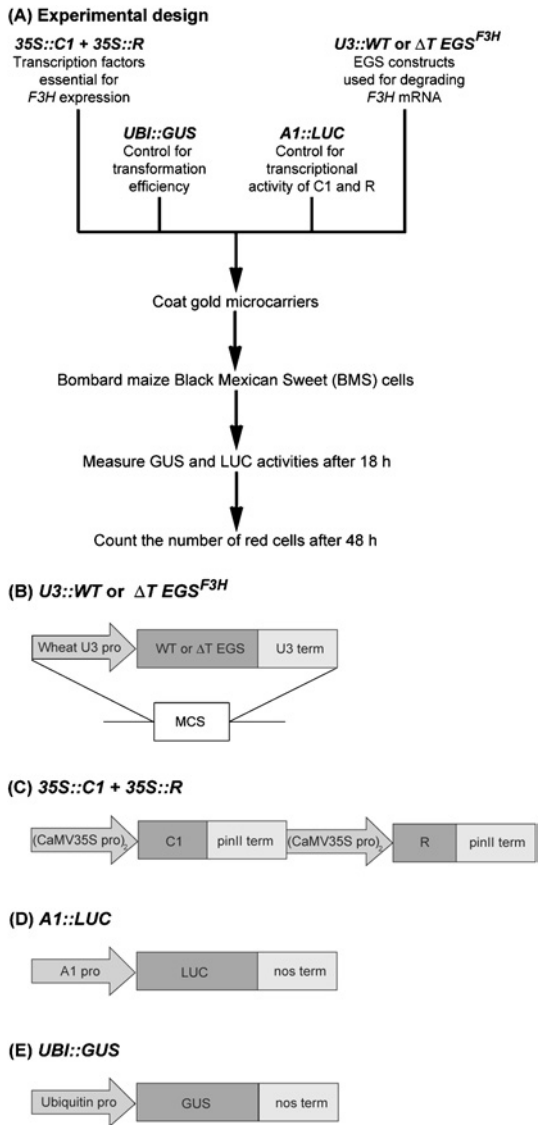


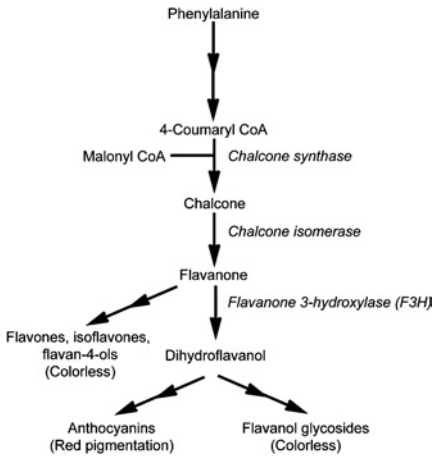
Figure 2 Details of the transient expression assay

(**A**) An outline of the experimental protocol. A complete description is provided elsewhere [17]. (**B**–**E**) Maps of the constructs used in this study. (CaMV35S pro)$_2$, two tandem 35 S promoter sequences; pro, promoter; term, terminator; UBI, ubiquitin.

pigments [18]. From an experimental perspective, accumulation of anthocyanins is cell-autonomous and provides a facile and stable visual phenotype (red coloration) for rapid screening [19]. Moreover, since *F3H* mutants have not yet been identified in maize, there is a need for acquiring maize plants with loss of F3H function by either gene knockout or knockdown strategies.

Selection of target site and design of EGSs

The steps involved in the EGS-mediated gene-inactivation technology are to (i) identify the single-stranded regions in the 5′ portion of the target mRNA accessible to EGS binding, (ii) design EGSs which, when complexed with the mRNA, will generate a non-natural substrate for plant RNase P, (iii) clone EGSs under the control of appropriate promoters for expression *in vivo*, and (iv) introduce EGSs into appropriate hosts and assay



Scheme 1 Flavonoid biosynthetic pathway

F3H activity is essential for the synthesis of flavanol glycosides and anthocyanins, which lend the red pigmentation, a phenotype exploited in the present study.

for the level of target gene expression by evaluating an appropriate phenotype. The complete experimental protocol for RNase P-mediated degradation of mRNAs in plants has been described elsewhere [16].

As the goal is to eliminate synthesis of the encoded protein, the EGS-mediated cleavage in the mRNA should be positioned close to the start codon to ensure complete non-translatability. By employing standard RNase T1 mapping and computer-aided secondary structure predictions of the F3H mRNA [16], we identified a single-stranded region near the start codon as an ideal target for EGS binding and subsequent cleavage by RNase P (Figure 1B).

To discriminate between RNase P- and antisense-mediated decrease in gene expression, our experiments used a *WT* and its corresponding disabled mutant $\Delta T\ EGS^{F3H}$ (Figures 1C and 1D). The $WT\ EGS^{F3H}$, upon hybridizing to its target mRNA, is expected to generate a bipartite structure that elicits cleavage by RNase P. In contrast, the $\Delta T\ EGS^{F3H}$, albeit having the regions complementary to the target mRNA, should generate an mRNA–EGS complex that is less susceptible to RNase P cleavage due to a deletion of the region equivalent to the T stem-loop in the ptRNA substrate, shown to be necessary for RNase P cleavage [20].

Expression of EGSs *in vivo*

To test the EGS RNAs *in vivo*, the corresponding genes were cloned under the control of a wheat *U3 snRNA* promoter known to be active in maize protoplasts for the generation of small RNAs [21]. Indeed, our results confirm the utility of this promoter for the expression of the EGS genes in maize BMS cells. *U3 snRNA* is transcribed by pol III (RNA polymerase III) in plants, and its promoter was chosen for the expression of EGSs rather than a pol II promoter for several reasons [11–13,21–23]. First, the regulatory elements in this pol III promoter reside entirely upstream of the coding regions of the gene. Secondly, the transcriptional activity of pol III promoters is severalfold higher than that of pol II promoters. Thirdly, the transcription start site of pol III promoters is better determined than that for pol II. Lastly, previous studies in mammalian cells have successfully disrupted gene expression using EGSs whose expression was driven by pol III promoters [6,12].

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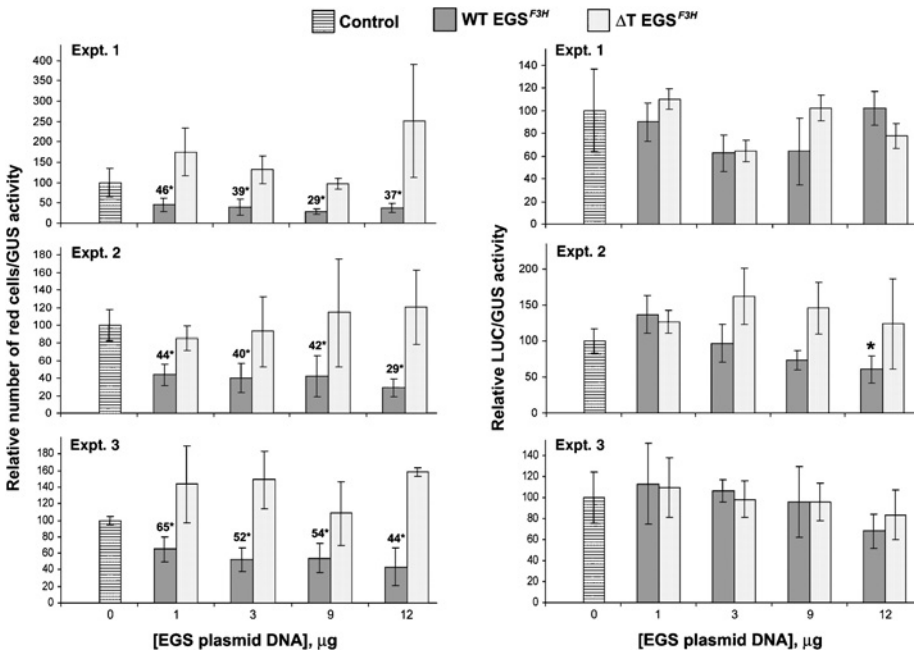

Figure 3 Extent of F3H suppression resulting from RNase P-mediated cleavage of F3H mRNA

The number of red-coloured cells/GUS activity reflects the levels of normalized F3H activity (left-hand panels), while the ratio of LUC/GUS activity represents the bombardment efficiency (right-hand panels). The values provided are relative to the control (i.e. absence of EGS), which is standardized to 100. The means ± S.D. were calculated using at least triplicates from each independent experiment. $*P < 0.05$ compared with the control (Student's *t* test).

Assessment of the efficacy of EGSs to disrupt *F3H in vivo*

In maize, several genes of the anthocyanin biosynthetic pathway, including *F3H*, are regulated by two transcription activators, C1 and R, which belong to the MYB and bHLH (basic helix–loop–helix) families respectively [24]. Since these activators are not expressed in BMS cells, these cells are colourless. However, when BMS cells are transformed with the *C1* and *R* genes, under the control of the cauliflower-mosaic-virus 35 S promoter (*35S::C1* + *35S::R*), the cells display red-coloured anthocyanin pigments [17,24]. If introduction of the *WT EGS^{F3H}* gene (*U3::WT EGS^{F3H}*) into *C1*- and *R*-transformed BMS cells by microparticle bombardment results in a depletion of F3H due to RNase P-mediated cleavage of the F3H mRNA, formation of the red pigment should be inhibited. A decrease in the number of red-coloured cells would therefore reflect the extent of reduction in the endogenous levels of F3H mRNA.

In several independent BMS bombardment experiments, we observed that introduction of the *WT EGS^{F3H}* plasmid DNA decreased the number of red-coloured cells to 29 % of that observed in cells lacking EGSs (referred to as the control; Figure 3). In contrast with the suppression observed with *WT EGS^{F3H}*, no decrease in the number of anthocyanin-accumulating cells was evident in cells bombarded with the *ΔT EGS^{F3H}* plasmid DNA at any amount (Figure 3, left-hand panels). This disabled EGS is not expected to induce RNase P-mediated cleavage of *F3H* mRNA. Any decrease in expression of target gene in cells bombarded with the *ΔT EGS^{F3H}* may be attributable to antisense or RNAi effects. As evident from the results shown in Figure 3, the contribution, if any, of antisense or RNAi to the disruption of *F3H* expression in these experiments was minimal.

As a means to normalize differences in the bombardment efficiency between samples, the *UBI::GUS* plasmid was included and the resulting β-glucuronidase (GUS) activity was used to normalize the red-coloured-cell numbers. Student's *t* test confirmed that, in three independent experiments (Figure 3, left-hand panels), the mean values for the number of red-coloured-cells/GUS activity obtained with the *WT EGS^{F3H}* were significantly different from those of the control (i.e. absence of EGS). This was not the case for the mutant *ΔT EGS^{F3H}* (Figure 3, left-hand panels).

To verify that the C1 and R regulators expressed from the introduced constructs were functional to similar levels in each bombardment, we included an *A1::LUC* control in which luciferase (LUC) activity results only when C1 and R activate transcription from the *A1* promoter. The maize *A1* gene encodes DFR (dihydroflavanol reductase), an enzyme downstream of F3H in the anthocyanin biosynthetic pathway. Like *F3H*, *DFR* is also subject to transcriptional activation by C1 and R. Since the mean of the LUC/GUS ratios was not significantly different in the various bombardments (Figure 3, right-hand panels), the highly reproducible decrease in red-coloured-cell numbers observed in the *WT EGS^{F3H}* bombardment experiments is not attributable to lower bombardment efficiencies or lack of functional transcriptional activators. Moreover, this decrease is not due to some unforeseen negative-feedback transcriptional-control mechanism involving C1 and R, since this would also have adversely affected *A1::LUC* expression, which is clearly not the case. Taken together, the effect of the *WT EGS^{F3H}* is likely to be post-transcriptional and results from RNase P-mediated degradation of the F3H mRNA. Since the low number of anthocyanin-accumulating cells in the transient experiments do not permit detailed molecular analyses, direct evidence for a decrease in F3H mRNA levels will have to await transgenic plants expressing the WT EGS^{F3H} RNA, which currently under investigation in our laboratory.

Conclusions and prognosis

Results from our transient expression studies using maize calli support the use of RNase P-based targeted degradation of

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mRNAs as a tool for engineering loss of gene function in plants. Since currently available post-transcriptional gene silencing methods have reportedly encountered the problems of off-target suppression [25] and variability in efficacy due to physical factors such as temperature [26] or poorly understood biological mechanisms [27,28], new approaches such as the EGS strategy for disrupting gene function might find utility in the post-genomics era.

While transient assays permit facile and rapid experimentation, certain features of these assays probably underestimate the efficacy of gene knockdown methods. For instance, two co-bombarded plasmids may not get delivered at the intended proportions to the same target cell or may not be expressed simultaneously due to differences in the timing of the activity of the promoters being used. Perhaps this might help to explain why we observe only a maximum of 71% suppression compared with approx. 90%, which was reported in some mammalian cells that stably expressed EGSs [11,12].

Although the EGS method might perform better *in planta* than one might anticipate from our transient assay results, it is important to appreciate that even a decrease of 70% protein levels is adequate to begin establishing gene–function relationships or for use in metabolic engineering applications. One study that employed RNAi for disrupting expression of a transcription factor [29] and another that employed the EGS approach for down-regulating the mRNA for a neuronal receptor [13] were both able to elicit the expected phenotypic alterations even when gene expression was reduced by only 50–60%. Moreover, it has been documented that metabolic flux alterations can be accomplished even with an approx. 30% change in a biological activity [30].

While ectopic expression of key enzymes in a biosynthetic pathway, and/or transcription factors that modulate expression of such enzymes, could be employed as a strategy for manipulating secondary metabolism [24], such an objective is frequently thwarted by the presence of a competing enzyme that shares a substrate with the enzyme whose expression is being enhanced artificially [31,32]. This obstacle could be circumvented by combining gene activation of the desired enzyme with suppression of the competing enzyme.

There is considerable interest in altering the activities of enzymes in the flavonoid biosynthetic pathway to facilitate the generation of pharmaceutically important products such as isoflavone phyto-oestrogens which display a wide range of chemopreventive activities against certain cancers, heart disease and osteoporosis [31,32]. An examination of the flavonoid biosynthetic pathway reveals that increased metabolic flux towards isoflavones could be accomplished by coupling the activation of rate-limiting enzymes for isoflavone synthesis with concomitant prevention of the F3H-mediated shunt towards anthocyanins (Scheme 1). Indeed, previous attempts to engineer the flavonoid biosynthetic pathway in *Arabidopsis* and soybean by gene overexpression alone were less successful than those which employed the two-pronged approach described above [31,32]. Our EGS-based suppression of *F3H*, in conjunction with activation of genes encoding enzymes in the isoflavone pathway, might facilitate production of high levels of isoflavones in maize.

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