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Trans-acting small interfering RNA4: key to nutraceutical synthesis in 1 grape development?

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Abstract

The facility and versatility of microRNAs (miRNAs) to evolve and change likely underlies how they have become dominant constituents of eukaryotic genomes. In this opinion article I propose that *trans-acting small interfering RNA gene 4* (*TAS4*) evolution may be important for biosynthesis of polyphenolics, arbuscular symbiosis, and bacterial pathogen etiologies. Expression-based and phylogenetic evidence shows that *TAS4* targets two novel grape (*Vitis vinifera* L.) MYB transcription factors (*VvMYBA6*, *VvMYBA7*) that spawn phased siRNAs and likely function in nutraceutical bioflavonoid biosynthesis and fruit development. Characterization of the molecular mechanisms of *TAS4* control of plant development and integration into biotic and abiotic stress- and nutrient signaling regulatory networks has applicability to molecular breeding and development of strategies for engineering healthier foods.

Functions of *TAS* loci

miRNAs and siRNAs are the specificity “guide” RNAs for nucleases of the ARGONAUTE (AGO) class which cleave or otherwise repress mRNAs in a nucleotide sequence-specific manner [1, 2]. *TAS* transcripts drive antisense transcription and feedback loops that amplify the production of siRNAs that in turn negatively regulate target gene expression in *trans*, an epigenetic process loosely described as “transitivity” important for viral defense [3, 4]. There are four known *TAS* genes- miR173 directs *TAS1* and *TAS2* siRNA biogenesis (unique to Arabidopsis) [5], whereas miR828 targets *TAS4* transcripts and miR390 initiates *TAS3* *trans*-acting small RNAs (tasiRNAs) that are deeply conserved in plants [3, 6]. The functional significance of *TAS3a-c* loci in leaf polarity specification and morphogenesis has been established [7], and is suggested for *TAS1a-c/2* as a means of dosage compensation by network repression of the large family of *PENTATRICOPEPTIDE REPEAT* genes in *Arabidopsis* (*Arabidopsis thaliana*) [8]. The functions of novel *TAS* candidates in rice (*Oryza sativa*) [9–11], soybean (*Glycine max*) [12], grape [13], tobacco (*Nicotiana tabacum*) [14, 15], and Norway spruce (*Picea abies*) [16] are not known. *TAS* loci, like *MIRNA* genes, have evolved independently at different times [17], and the derived tasiRNAs share some biosynthetic steps with miRNAs [18].

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Supplementary data

Supplementary data associated with this article can be found at doi:XXXXXXX'

The fundamental difference between the miRNA and tasiRNA pathways is the origins of the respective small (~21–22 nt) RNAs: *MIRNAs* are loci whose transcripts can fold back on themselves into imperfect hairpin structures (pre-miRNAs) that are “diced” into duplexes with two nt 3’ overhangs by DICER-LIKE1 (DCL1), whereas *TAS* gene transcripts are copied after the action of a specific miRNA into long perfectly complementary dsRNAs diced by DCL4 to produce tasiRNAs, which like miRNAs can target genes for silencing. The activity of tasiRNAs as repressors of endogenous target mRNAs in Arabidopsis and maize (*Zea mays*) involves AGO1 as well as AGO7 (for *TAS3a-c*) [19, 20] and is triggered by a two miRNA hit model [4, 21, 22] or through initial targeting by longer 22 nt miRNAs [23, 24] or a bulged miRNA-miRNA* duplex [25]. In the *TAS*-specific pathway of RNAi, RNA DEPENDENT RNA POLYMERASE6 (RDR6) copies the miRNA-cleaved *TAS* product into dsRNA with the help of plant-specific SUPPRESSOR OF GENE SILENCING3 (SGS3) [26]. There are distinct cellular pools of AGO1 loaded with miRNAs versus siRNAs, revealed by *in planta* studies of viral RNA silencing suppressor proteins [27] which underscore the evolutionary complexity of plant RNAi pathways and their perturbation by biotic stresses.

The recent discovery of circular non-coding RNAs (ncRNAs) that function as “miRNA sponges” in animals [28], and miRNA “target mimic” ncRNAs in plants [29–31] raise the profile of *TAS* genes because these classes of ncRNAs are all examples of systemic innovations (e.g. signals, decoys, guides, scaffolds) that impact the circuit topology of miRNAs [32]. Unexpected positive correlations observed between expression of miRNAs and their predicted targets [33] support the notion that there are other processes (e.g. feedback loops and RNA decoys) that can impact miRNAs as canonical negative regulators. Intriguingly, predicted target mimics of miR858 [34], *MYBL2* and *MYB34*, regulate biosynthesis of anthocyanin and aromatic amino acid-derived indole glucosinolates, respectively [35] that account for ~20% of the carbon fixed by plants to make secondary metabolite anthocyanins, defensive phytoalexins, and structural lignin. As elaborated below, certain *MYB* transcription factor genes are conserved key effectors of these pathways and targeted directly by *TAS4*, which is also subject to a homeostatic autoregulatory feedback loop by the targeted MYBs [36, 37].

***TAS4*, miRNAs, and MYBs**

TAS4 was discovered in *Arabidopsis* [6] where it generates a ~1kb long ncRNA spawning phased (21 nt registers) sense and antisense small interfering RNAs (*ta*-siRNAs) triggered by miR828, a member of the class of 22 nt DCL1-dependent miRNAs that serve as specificity determinants for the *TAS* pathway of RNAi. In *Arabidopsis*, the tasiRNA *TAS4*-3’-D4(–) (an antisense species four registers downstream from the miR828 trigger) targets a set of *MYB* transcription factors *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1/MYB75*), *PAP2/MYB90*, and *MYB113* that regulate the anthocyanin and lignin biosynthesis pathways [38, 39] and is inducible by phosphate starvation, sugars, and the plant hormone abscisic acid (ABA) [36, 37] important for seed development and stress responses including anthocyanin production [40, 41]. The R2R3 MYB class of DNA-binding transcription factors is the most abundant superfamily in plants (126 members in *Arabidopsis*) [42] and several narrow MYB clades are targeted by miR828/*TAS4* and miR858, both of which are involved in flavonoid and lignin biosynthesis [6, 43–46]. The convergence of miR828/*TAS4*, miR858, and possibly miR858 endogenous target mimics on only this handful of MYBs involved in responses to and crosstalk between ABA, sugar, and phosphate signals leading to nutraceutical/lignin synthesis is remarkable from an evolutionary standpoint and warrants further study.

Because miRNAs and *TAS*s act dominantly, they are a particularly good subject for studying the fitness landscape of interactions and genetic robustness in nature and at the bench. By their intrinsic nature as nodes in networks, siRNAs can lend themselves to biotechnological applications such as functional genomics and manipulating productivity and quality in specialty crops, forestry products, and horticultural species by RNAi. Accelerated breeding of crops and trees might be immediately achieved by selecting elite progeny with desirable target haplotypes (e.g. associated with pathogen resistance) regulated by *TAS4* siRNAs and/or miR828/miR858, and eventual cogent engineering of traits such as fruit flavor and nutraceutical content, secondary metabolites for biomass feedstocks, beneficial microbe– plant interactions, and improved stress responses and productivity through better management of fertilizer and greater efficiency in phosphate uptake.

In this opinion article I present some direct and phylogenetic evidence that *TAS4* may be a key effector controlling flavonoid synthesis, fruit development, and symptoms of a specific bacterial disease, at least in grape. The observations are drawn from genome-wide analyses across angiosperm clades and approach the key question in evolution- how high resiliency might come about through evolution that changes circuit topology gradually, one interaction at a time [47]. Bioflavonoid regulation through target MYBs by *TAS4* siRNAs and miRNAs may be corollary to sugar deposition in flowers and fruits- two prime examples of evolution through facultative symbiosis, whereby the abundance and nutritional compositions of plant organs aid insect pollination and animal seed dispersal, respectively. Because bioflavonoids have salutary effects on cardiovascular function, cancer prevention [48], and neurological processes [49], I speculate that *TAS4* may have co-evolved with frugivores because of their derived fitness. Supporting this notion is that mammals generally, and birds to some degree, show a trend of brain expansion over evolutionary time that is absent in other vertebrates [50–53]. Much work remains to substantiate such an idea- for example, can nutraceuticals increase the reproductive longevity of species? What is the relationship between nutraceutical consumption and coloration in birds [54]?

TAS4 evolution

In addition to *TAS4*-3'-D4(-) targeting of *MYB113*, miR828 also targets *MYB113* directly, suggesting a close evolutionary relationship between *MYB75/90/113*, miR828, and *TAS4* that function in a regulatory network or hierarchy that includes autoregulation [36, 37]. miR828 is the trigger for production of *TAS4* siRNAs, and was originally thought to be unique to *Arabidopsis*, but has subsequently been found in grape [55], poplar (*Populus trichocarpa*) [56], cotton (*Gossypium hirsutum*) [57], cabbage (*Brassica rapa*) [58], flax (*Linum usitatissimum*) [59], soybean (*Glycine max*) [21], common bean (*Phaseolus vulgaris*) [60], clary sage (*Salvia sclarea*) [61], melon (*Cucumis melo*) [62], rose (*Rosa multiflora*) [63], tomato (*Solanum lycopersicum*) [64], tobacco (*Nicotiana tabacum* and *N. attenuata*) [65, 66], cocoa (*Theobroma cacao*) [67], Chinese apricot (*Prunus mume*) [68, 69], peach (*Prunus persica*) [70, 71], citrus (*Citrus sinensis*) [72], apple (*Malus domestica*) [73], hickory (*Carya cathayensis*) [74], cassava (*Manihot esculenta*) [75], Brazilian cherry (*Eugenia uniflora*) [76], Chinese tulip tree (*Liriodendron chinense*) [77], and Red Sea mangrove (*Avicennia marina*) [78]. Figure 1 shows a phylogram of the evolutionary relationships for eudicot species which have evidence of *TAS4* in their genomes. It is intriguing that all of these species produce flavonoid nutraceuticals, framing the question of evolutionary significance of miR828 and *TAS4*. We previously found candidate *MIR828* sequences in gymnosperms and *Trillium camschatcense*, a basal monocot with broad, thin, soft leaves, net venation, and fleshy fruits, and suggested that miR828 or *MYB* pseudogenes may have evolved into *TAS4* by a neofunctionalization of an ancient *MIR828* possibly important for the diversification of dicotyledonous flowering plants, which occurred ~100MYa [79] concurrent with diversification of mammals and frugivory [80]. Three other

groups also found miR828 and miR858 in Chinese fir (*Cunninghamia lanceolata*) [81] and *Taxus mairei* [82] or predicted miR828 and miR858 and *MYB* targets in Norway spruce (*Picea abies*) [83]. We predicted that grape *MYBA6*, an uncharacterized gene, was subject to regulation by Vv *TAS4*, based on conservation at the protein level of the putative TAS4-3'-D4(-) binding site in the mRNA [37]. Figure 2 shows evidence of selective sweep for the miR828 binding site of *TAS4* and for the D4 species of tasi-RNA duplex from numerous eudicot species, supporting the hypothesis that tasiRNA VvTAS4-3'D4(-) plays an important functional role in fitness of eudicots, namely the targeting of *MYBs* involved in bioflavonoid and secondary metabolite biosynthesis.

Transitivity of *MYB* targets associated with miR828 and *TAS4*

Interestingly, clusters of “phased siRNAs” (phasiRNAs) [21] map to *MYB* targets in grape [13, 16], poplar [56], sweet potato (*Ipomoea batatas*) [84], apple [73], peach [71], soybean [21], and cotton (Guan X, Pang M, Chen ZJ. Annu Meeting Amer Soc Plant Biol, P16052. http://c.ymcdn.com/sites/my.aspb.org/resource/resmgr/Docs/Final_Program_Book.pdf) which have been attributed to miR828 activity, but the functional significance of this transitivity is unknown and evidence for miR828 causality is lacking. Recent reports documented miR828-directed cleavage of both *TAS4* and numerous *MYBs* in apple [73] and peach [71] and invoked the “two-hit model” [22] to explain observed transitivity of *MYBs*, based on weak predicted binding affinity of a different miR858 [73]. Below I provide evidence from publicly available datasets that the two hit model does not explain transitivity for two novel *MYBs* in grape involved in polyphenolic nutraceutical synthesis, but rather is controlled by *TAS4*.

A conserved *TAS4* tasiRNA silences Vv*MYBA6* and Vv*MYBA7*

By computational analysis of sRNA-Seq datasets from a wild species of grape, *Vitis amurensis* Rupr [85], Pinot noir variety of *V. vinifera* [86], and Merlot leaf, flower, and fruit libraries (GSE28755), compelling evidence of production of phasiRNAs from Vv*MYBA6* and *MYBA7* triggered by TAS4b-3'D4(-) has been obtained (see the supplementary material online Figure S1). Supporting evidence for production of perfect match tasiRNAs to Vv*MYBA7* phases 0 and 4 (not shown) was found in a Pinot noir degradome library [86], and has been recently documented for Vv*MYBA7* and Vv*MYBA7* in a Merlot variety library (GSM803800) [16]. miR828 likely does not function on these target mRNAs due to several mismatches at “seed” position 7–11 in plants (see the supplementary material online Figure S1B) which would preclude binding activity based on functional and structural analyses [87, 88]. Almost all the perfect match 21 nt siRNAs mapping to the sense strand of Vv*MYBA6* and *MYBA7* mRNAs are in perfect register downstream (3') to the predicted cleavage site of TAS4-3'D4(-) at “phase 0” (see the supplementary material online Figure S1A). Antisense reads for phases 3, 4, and 7 are found for Vv*MYBA6* in two libraries (Amur and tendril), whereas phases 4, 8, and 11 are found for Vv*MYBA7* in three (tendril, fruit, flower) libraries (data not shown). It is interesting that for a majority of these antisense species, sense species are not observed (see the supplementary material online Figure S1A), consistent with the notion that the antisense phasiRNAs are loaded preferentially into an AGO or otherwise stabilized at the expense of the sense phasiRNAs. An upstream sense siRNA at phase-8 (see the supplementary material online Figure S1A) suggests that dsRNA can be generated and diced in both directions from the 3'D4(-) trigger, as has been shown in *Arabidopsis* [89]. Taken together, these observations show that *TAS4* functions in grape to silence *MYBA6* and *MYBA7* in leaves, flowers, and fruits and suggest a conserved molecular mechanism for the observed transitivity of *MYB* targets in numerous other dicots which have *TAS4* (Figure. 1).

Expression patterns of *TAS4abc* tasiRNAs elucidate gene activities

There exist three *TAS4* loci in grape (Figure 2) which generate isoforms of *TAS4*-3'D4(-) with implications for differential *MYB* cleavage activities (see the supplementary material online Figure S1B). *TAS4a* and *TAS4b* map to chr. 14 and have EST evidence for expression, whereas Vvi-*TAS4c* was previously mis-annotated as Vvi-miR828b [55], a contention supported by its poor secondary hairpin structure (three gaps and three mismatches in the mature duplex; www.mirbase.org) and similar extended homologies to those observed between *Arabidopsis TAS4* and *MIR828* [37]. All three isoforms are expressed, based on identification of perfect-match unique siRNAs in eight different RNA-Seq libraries, with *TAS4c* expression being lower than *TAS4a* or *TAS4b* by > three orders of magnitude and on a par with miR828 and miR828* abundance (see the supplementary material online Figure S2C,D). The high reproducibility of abundances between libraries of the *TAS4b* species D1(+) and D4(-) ($r=0.78$ and 0.77 , respectively) compared to *TAS4a* D1(+) and D4(-) species ($r=-0.67$ and 0.20) supports the notion that *TAS4b*-3'D4(-) is the trigger for the observed Vv*MYBA6* and *MYBA7* tasiRNAs, consistent with a higher binding affinity of *TAS4b*-3'D4(-) over that of *TAS4a*-3'D4(-) (see the supplementary material online Figure S1B).

VvMYBA6 and VvMYBA7 functions in bioflavonoid synthesis

The novel Vv*MYBA6*, Vv*MYBA7*, and other MYBs targeted by miR828/*TAS4* comprise only ~7% of all R2R3 MYBs encoded in grape and ~2% of those in *Arabidopsis*. Those *MYB* targets of *TAS4*/miR828, remarkably, are most homologous [90] to those MYB transcription factors (*Fl*, *PA1/2*, Vv*MYBA1-3*) that have been shown to be effectors, respectively, of Flavonol, ProAnthocyanidin, and Anthocyanin pathways in apple [73], grape [91–95], and *Arabidopsis* (clades S5–S7) [96], the latter clade which is conspicuously absent (like miR828 and *TAS4*) from monocots [97]. Figure 3 shows a phylogram of *TAS4* and miR828 [86]-validated MYB targets in grape, peach, and *Arabidopsis* [6] plus an outlier MYB from maize most homologous to the S6 clade [97]. Interestingly, the *TAS4*-validated or -predicted targets in peach are more diverged from their nearest miR828-targeted paralogues, suggesting they have evolved novel functions related to nutraceutical metabolism. Identification of MYB proteins involved in the control of secondary metabolism and development within subgroups across model species have shown that homologues participate in the same processes [96]. The central positioning of Vv*MYBA6* and Vv*MYBA7* between S6 and S5/7/13 clades for Anthocyanin and Proanthocyanidin/Flavonol/Ligning regulation [96], respectively, supports their potential function in each of these pathways.

Correlative evidence for VvMYBA6 and VvMYBA7 functions

It is significant that non-small RNA targets Vv*MYBA1-3* isogenes clustered on Chr. 2 do not account for all observed anthocyanin phenotypes [98] or flavonoid pathways [94], suggesting Vv*MYBA6* and Vv*MYBA7* are effectors in a hierarchy of MYBs [96] controlling fruit pigment synthesis. Association mapping [99] demonstrated that only 62% of anthocyanin content variation could be attributed to the Vv*MYBA1-3* isogenes in a Syrah×Grenache F₁ pseudo-testcross. This finding is consistent with the hypothesis that other regulators, viz. Vv*MYBA6* and Vv*MYBA7* on Chr. 14 that are targets of Vvi-*TAS4b* may control véraison, the seed maturation phase in grape berry development that coincides with transition from acid to sugar accumulation, production of volatiles and antioxidant tannic pigments, and breakdown of chlorophyll and methoxypyrazines. Circumstantial evidence for *MYB* genes controlling fruit and leaf development are the pleiotropic phenotypes of Pinot Meunier sport “Samtrot,” which has glabrous apices and leaves, a well-known phenotype caused by *MYB* mutations in many species [90], lower fruit set, lower yields, and higher sugar content than compared to Pinot Meunier or Pinot noir [100].

Nutraceutical properties and biosynthesis Flavonoids (Latin: *flavus*, “yellow”) are secondary polyphenolic metabolites involved in many aspects of plant development, auxin transport [101], ABA biosynthesis [40], and structural reinforcement. They were called “vitamin P” (not to be confused with the current euphemism for the anti-depressants Prozac and Paxil) early in the last century, due to their effects on *permeability* of vascular capillaries. They contribute attractive colors to fruits and flowers that aid in seed and pollen dispersal, and adaptation to environmental conditions such as cold, phosphate starvation, UV stresses, pathogen attack, allelopathy, mediation of stigma–pollen interactions, and signaling to mutualist microbes for nitrogen fixation [102–104]. Regulation of flavonoid/ polyphenolic biosynthetic genes is tightly organized in a spatial and temporal way orchestrated by ternary complexes involving transcription factors from the R2R3-MYB, basic helix–loop–helix (bHLH), and WD40 classes [104–107]. Flavonoids, stilbenes, and polyphenolics (tannins) are components of fruit that impart color, aromas, and flavor enhancers associated with purported nutraceutical benefits (e.g. antioxidant and anti-inflammatory properties) and in-mouth tactile sensations (astringency). They comprise four main classes of compounds (Figure 4) whose biosynthesis during fruit development and stress response is mediated by genes encoding enzymes for committed branch pathways from 4-coumarate (the product of Phe catabolism by Phe Ammonia Lyase): flavonols by flavonol synthase (*FLS*) and UDP-glucuronic acid/galactose:flavonol-3-O-glucuronosyltransferases (*GT5/6*), anthocyanins by UDP-glucose:flavonoid 3-O-glucosyltransferases (*UGT*), and proanthocyanidins by leucoanthocyanidin reductase (*LAR*) and anthocyanidin reductase (*ANR*) [108, 109], which are also involved in lignin biosynthesis [110] important for stonefruit development [111]. Recent reports have shown that sugars and sunlight induce anthocyanin accumulation and flavanone 3-hydroxylase (F3H, an upstream enzyme in the flavonoid pathway) in grape berries [112, 113]. Water deficit increases stilbene metabolism [114]. Water stress at pre- and post-véraison stages affects anthocyanin composition differently, suggesting a differential regulation of genes involved in the last steps of anthocyanin biosynthesis pathway [115], possibly mediated by VvMYBA6, VvMYBA7, and/or other miR828/ miR858- targeted MYBs (Figure 4).

Potential environmental forces driving evolution of *TAS4* and target *MYBs*

Flowering plants presented Darwin with the most extreme exception to his strongly held notion *natura non facit saltum*, ‘nature does not make a leap’, resulting in him describing plant evolution as an “abominable mystery” [116]. Defense against pathogens is one of the most potent drivers of evolutionary change in host organisms. Recent reports show that like viruses, various types of fungi and bacterial pathogens have evolved RNAi suppressor proteins important for disease etiologies [117–119]. The bacterium *Xylella fastidiosa* (XF) is the cause of Pierce’s disease in grapes and is a major threat to fruit, nut, and coffee groves. Obvious symptoms are anthocyanin accumulation in concentric zones of leaves and in xylem sap, and shriveling of undeveloped berries [120]. XF infection results in significant and reproducible decreases in leaf elemental phosphorus in several species including grape [121]. Phosphorous is the second most limiting macronutrient to plants affecting crop quality and production; the classic symptom of inorganic phosphate (P_i) deficiency is anthocyanin accumulation. P_i treatment induces systemic acquired resistance to pathogens and shares some common early steps (RNAi?) with tobacco necrosis virus acquired resistance [122]. P_i deficiency causes delays in grape berry maturity and reduced berry size [123], but the molecular mechanisms are unknown.

In the context of XF disease symptoms of wilting and berry shrivel, it is interesting to note that vascular parenchyma cells at the boundary between xylem and phloem bundles are the site of drought-inducible ABA biosynthesis and transport [124] by the action of NINE-CIS-EPOXYCAROTENIOD DIOXYGENASES1/4 (NCED1/4), the rate-limiting step of ABA

biosynthesis, which is also up-regulated during berry development [125]. Transport of ABA, TAS4 tasiRNAs, and miRNAs through the vasculature may be important for the same physiological processes driving evolution of plants and their ecological partners/pathogens. For example, phosphorus deficiency marked by miR399 induction and movement through the vasculature [126] has been identified as an etiological link to citrus Huanglongbing disease [127], caused by the gram-negative bacterium *Candidatus Liberibacter*. Iron and phosphorus homeostasis are coupled [128], consistent with the notion that miRNAs regulating iron transport [119] and phosphate signaling could be targets of pathogens. Another functional insight into a longstanding problem of why mutants affecting sRNA biogenesis and activity are ABA hypersensitive [129] is the recent finding that ALTERED MERISTEM PROGRAM 1, a negative regulator of ABA and drought response [130, 131], mediates translational inhibition by miRNAs at the endoplasmic reticulum [132], which appears to be a site of action of AGOs [133–135]. Roles for ABA, P_i , and miR828/TAS4 in XF disease etiology have not been proposed. Since TAS4 expression is induced by phosphate starvation, sugars, and ABA in Arabidopsis [36, 37], I hypothesize that TAS4 plays a role in disease etiology of Pierce's disease and berry development by VvMYBA6 and VvMYBA7 regulation. Additional evidence is the mapping of a quantitative trait locus for Pierce's disease resistance [136] very near to VvMYBA6/7 and a validated miR828 MYB target GSVIVT01032467001 [86] on Chr. 14 (Figure 3). Taken together with the knowledge that microbes disrupt miRNA and RNAi pathways, the recent observations that Grapevine Leaf-Roll18 associated Virus infection increases miR828 accumulation [137], blocks berry development [125] and phenocopies symptoms of Pierce's disease [138] is strong correlative evidence of TAS4 and miR828 function in véraison and Pierce's disease, because viruses have evolved suppressor proteins that interfere with small RNA formation or activity [27].

I speculate that mycorrhizal arbuscular associations between plant roots and fungi, where the plant provides carbohydrates to the fungus in return for phosphate, may be evidence for the hypothesis of TAS4/miR828/MYB co-evolution related to Nodulation factor (NodD) induction by flavonoids secreted by hosts in Rhizobium symbiosis [139, 140]. Others have recently shown that several miRNA families function as master regulators of defense response by production of phasiRNAs [16, 21, 141] targeting the large family of NB-LRR genes, which along with MYBs are classified as PHAS loci based on the miR828-associated [13, 21, 56, 73, 84] and TAS4-triggered transitivity of VvMYBA6 and VvMYBA7 shown here. The diversity and conservation of PHAS loci across plant taxa, including soybean miR828 target MYBs [21, 142], and their loss in virus- and bacteria-infected tissues [141, 143] that results in pathogen susceptibility, demonstrates broad functions of miRNA PHAS effectors as master regulators [21]. This process amounts to exploitation by plants of pathogen-derived suppressors of RNAi to achieve inducible expression of defense- and/or development-related genes. It is remarkable that small RNA effectors DCL2, SGS3 (in legumes and soybean/grape, respectively), and AGO1 can spawn phasiRNAs themselves [16, 21, 144–146] and have acquired some miRNA target sites independently [21]. Furthermore, AGO2 and DCL1 are targeted by miR403 and miR162, respectively [3, 147], and DCL1 encodes MIR838 in one of its introns [6], underscoring the evolutionary importance of recruitment of RNAi into homeostatic feedback circuits and ncRNAs.

Implications for healthier food and sustainable agriculture

Detailed, genome-enabled understanding of gene networks and the life cycles of RNAs can be translated into tools and resources for development of crops that efficiently respond to local and changing conditions. Quality improvements depend on applying new genetic insights and new technologies to accelerate breeding through improved genotyping and phenotyping methods, and by increasing the available genetic diversity in germplasm [148,

149]. It takes years of breeding and testing to obtain a new grape variety and substantial space to grow out seedlings to a bearing year for phenotyping. Breeding is not widely practiced in viticulture because markets, traditions, and statutes dictate cultivar choice, thus varieties and clones lack recombination and the resultant opportunity to select for adaptability, e.g. Pierce's disease resistance. Molecular breeding can have significant impacts on specialty crops by: (i) applying deep knowledge from model species to reference genomes and expression datasets; (ii) facilitating optimal selection of parents and immediate selection of elite progeny with multiple desirable traits; (iii) circumventing biological and societal limits to genetic engineering; (iv) accessing abundant genetic variation, and (v) rapid linkage disequilibrium allowing single nucleotide polymorphisms of genes (e.g. *VvMYBA6* and *VvMYBA7*) to be directly associated with phenotypes.

In the post-genomics era, the broad goals of translational science are to integrate growth and development processes to the environmental cues that shape an individual or species by evolution. Suppression and induction of the host sRNA silencing systems likely plays some fundamental role in plant-microbe interactions [21, 119, 141, 143, 150]. The availability of reference gymnosperm genomes such as *P. abies* [151], *P. glauca* [152], *Pinus contorta*, and *Pinus resinosa* [37] will facilitate future studies on evolution of *MIR828*, *TAS4*, *MIR858* [16] and possible applications of the target genes to manipulate lignin biosynthesis. Interestingly, a recent report showed the possible convergence between *P. abies*, *Amborella*, *Medicago*, poplar, cotton, and grape for silencing of *NBS-LRR* genes (and conservation of *TAS4*/miR828-targeted *MYBs*; my observations) by production of 21 nt phasiRNAs [16], demonstrating the deep evolutionary forces that have shaped the function of miRNAs and tasiRNAs as master regulators of *MYB* and other large gene families in plants. Knowledge of the molecular mechanisms of *TAS4* function will provide cogent means to manipulate productivity and quality in specialty crops, forestry products, and horticultural species by RNAi and transgenic technologies. The *TAS4* mechanism may be important for other plant-pathogen interactions and may have significance for plant-microbe and frugivore-plant co-evolution. Detailed characterization of miR828 and *TAS4* function in seed plants will reveal fundamental processes and mechanisms in plant biology which may change our understanding of healthy food. The recent publication of the NIH Microbiome Project [153] nicely frames the systems approach to transform understanding of the nature of human relationships (commensal, mutual, or parasitic) with our flora. I submit that "flora" includes those plants that animals eat and that intestinal microbiomes process for immediate and generational fitness. For example, it is now a tractable question to address: how did evolution adapt fermentation of flavonoid pathways that transform our preferred nutraceutical staples of cocoa and grape?

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Evolution of TAS4 important for eudicot fitness, possibly driven by animal frugivory
- A TAS4 siRNA targets two novel grape MYBs important for nutraceutical biosynthesis
- Transitivity of TAS4- and miR828-targeted MYBs suggests a master regulatory role
- MYB targets of TAS4 are potential effectors of fruit development and microbial disease symptoms
- Biotech applications of TAS4 have potential for healthier foods and biomass feedstocks

Figure 1.

Phylogram (www.phytozome.org) from completed draft genomes showing evolutionary relationships of eudicot species having an expressed *TAS4* paralogue (tick). *TAS4* sequences were previously identified for *Populus*, *Euphorbia*, *Mimulus* [37], *P. persica* [71] and *M. domestica* [73]). Sequences (see Figure 2) of *TAS4* paralogues were obtained by BLASTing *TAS4/AT3G25795* (n.t. 870–980) or *TAS4-3'-D4(-)* into GenBank plant Expressed Sequence Tag (EST) database (<http://www.ncbi.nlm.nih.gov>).

Evidence of selective sweep for expressed TAS4-3'D4, supporting *TAS4* function important for fitness of eudicots. Sequence alignment of *TAS4* paralogues revealed by BLASTing TAS4/AT3G25795 (n.t. 870–980) or TAS4-3'-D4(-) into GenBank plant EST database; IDs shown except *P. persica*= scaffold1 coords (www.phytozome.org), Nbe/Spi scaffolds/contigs (<http://solgenomics.net>; Nbe-TAS4a_Scf25079402_1100; Nbe-TAS4b_Scf24955202_1557rc; Spi-TAS4_contig_6644804rc), and Nta GSS reads (<http://www.pngg.org>; Nta-TAS4a_CHO_OF5007xj16; Nta-TAS4b_CHO_OF5124xc17rc). Alignments are color-coded on the confidence of alignment by T-Coffee (www.tcoffee.org). The putative miR828 binding site (upper block) and TAS4-3'D4(-)-generating site (lower block) are underscored with black lines. Asterisks (*) show consensus (cons). Abbreviations: Ach, *Actinidia chinensis* (kiwi); At, *Arabidopsis thaliana*; Bol, *Brassica oleracea*; Csi, *Citrus sinensis*; Mdo, *Malus domestica*; Nbe, *Nicotiana benthamiana*; Nta, *Nicotiana tabacum*; Ppe, *Prunus persica*; Sly, *Solanum lycopersicum*; Spi, *Solanum pimpinellifolium*; Tca, *Theobroma cacao*; Vv, *Vitis vinifera* (VviTAS4c= chr1..2961681rc).

Figure 3.

Phylogram of validated miR828 (☺)- and/or *TAS4*-targeted (\$) grape (purple) or predicted peach [71] (orange) and *Arabidopsis* (blue) MYB proteins. Clustering of subgroups across species provides evidence of conserved MYB functions for synthesis of Proanthocyanidins [subgroup 5 (S5)], Anthocyanins (subgroup 6), and lignins (subgroup S13) based on functional analyses [96]. The subgroup functional annotations for maize (Zm prefix; outgroup gene most similar to subgroup 6), grape (Vv or GSVIVT prefixes [90]), peach (Ppa), and *Arabidopsis* (At) MYBs are as described [71, 90, 96, 97]. Likelihood metric of node lengths is shown in red.

Figure 4.

Simplified flavonoid biosynthetic pathway showing enzymatic steps specific to the biosynthesis of nutraceutical stilbenes (RS), flavonols (FLS), proanthocyanidins (and lignin [110, 111], HCT) (LAR and ANR), and anthocyanins (UFGT). *Arabidopsis*, grape, and peach MYBs subject to miR828 [71, 86], miR858 [6, 43–46], or *TAS4* regulation and inferred (from Figure 3) to regulate known [96, 110, 111] or predicted polyphenolic pathways are shown. eTM: predicted endogenous miR858 Target Mimics [34]. See supplementary material online for description of miR858 results. Figure modified from Cohen et al [109].

MYBs: miRNA/*TAS4*

AtMYB34: miR858 eTM?

AtMYB83: miR858

Three PpMYBs: miR828

Two PpMYBs: *TAS4*?

AtMYB11,12,111: miR858

AtMYB123/TT2: miR858

Five VvMYBs: miR828

AtMYBL2: miR858 eTM?

AtMYB113: miR828, *TAS4*

AtMYB75,90: *TAS4*

VvMYBA6,7: *TAS4*