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Opinion

Cell PRESS

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

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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 small interfering RNAs (siRNAs) which probably 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 the 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].

The fundamental difference between the miRNA and tasiRNA pathways is the origins of the respective small (~21–22 nt) RNAs: *MIRNA*s 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 double-stranded (ds)RNAs diced by DCL4 to produce tasiRNAs, which like miRNAs can target protein-coding 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 RNA interference (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], raises the profile of *TAS* genes because these classes of ncRNAs are all examples of systemic innovations (e.g., signals, decoys, guides, scaffolds) that impact upon 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 upon miRNAs as canonical negative regulators. Intriguingly, predicted target mimics of miR858 [34], *MYBL2* and *MYB34*, regulate the biosynthesis of

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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, particular *MYB* transcription factor genes are conserved key effectors of these pathways and are 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 ~1 kb ncRNA that spawns phased (21 nt registers) sense and antisense small interfering RNAs (tasiRNAs) 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 are 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 a 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 neofunctionalization of an ancient *MIR828* possibly important for the diversification of dicotyledonous flowering plants which occurred ~100 million years ago [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

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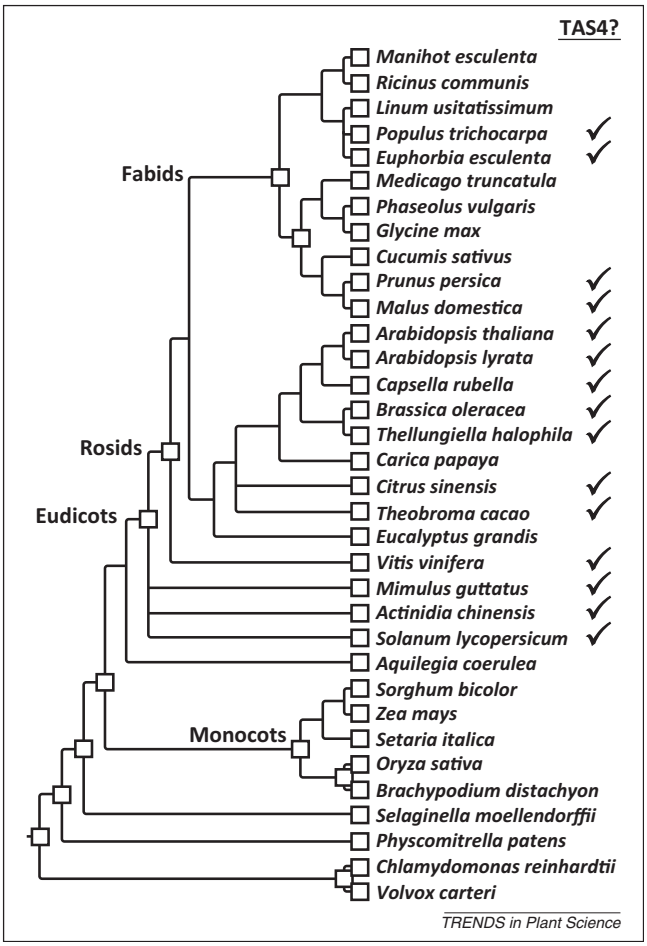

Figure 1. Phylogram (www.phytozome.org) from completed draft genomes showing evolutionary relationships of eudicot species having an expressed *TAS4* paralog (tick). *TAS4* sequences were previously identified for *Populus, Euphorbia, Mimulus* [37], *Prunus persica* [71], and *Malus domestica* [73]). Sequences (see Figure 2) of *TAS4* paralogs were obtained using BLAST (basic local alignment search tool) analysis for *TAS4/AT3G25795* (nt 870–980) or TAS4-3′-D4(–) in the GenBank plant expressed sequence tag (EST) database (http://www.ncbi.nlm.nih.gov).

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 tasiRNA duplex from numerous eudicot species, supporting the hypothesis that tasiRNA TAS4-3′D4(–) plays an important functional role in fitness of eudicots, namely the targeting of *MYB*s 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. *et al.*, *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 *MYB*s in apple [73] and peach [71], and invoked the 'two-hit model' [22] to explain observed transitivity of *MYB*s, 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 instead is controlled by *TAS4*.

A conserved TAS4 tasiRNA silences VvMYBA6 and VvMYBA7

By computational analysis of small RNA sequencing (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 *VvMYBA6* and *MYBA7* triggered by TAS4b-3′D4(–) has been obtained (see Figure S1 in the supplementary material online). Supporting evidence for production of perfect match tasiRNAs to *VvMYBA7* phases 0 and 4 (not shown) was found in a Pinot noir degradome library [86], and has been documented recently for *VvMYBA7* and *VvMYBA7* in a Merlot variety library (GSM803800) [16]. miR828 likely does not function on these target mRNAs owing to several mismatches at 'seed' position 7–11 in plants (see Figure S1B in the supplementary material online) 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 *VvMYBA6* and *MYBA7* mRNAs are in perfect register downstream (3′) to the predicted cleavage site of TAS4-3′D4(–) at 'phase 0' (see Figure S1A in the supplementary material online). Antisense reads for phases 3, 4, and 7 are found for *VvMYBA6* in two libraries (Amur and tendril), whereas phases 4, 8, and 11 are found for *VvMYBA7* 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 Figure S1A in the supplementary material online), 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 Figure S1A in the supplementary material online) 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 are three *TAS4* loci in grape (Figure 2) which generate isoforms of TAS4-3′D4(–) with implications for differential *MYB* cleavage activities (see Figure S1B in the supplementary material online). *TAS4a* and *TAS4b* map to chromosome (Chr.) 14 and have expressed sequence tag (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;

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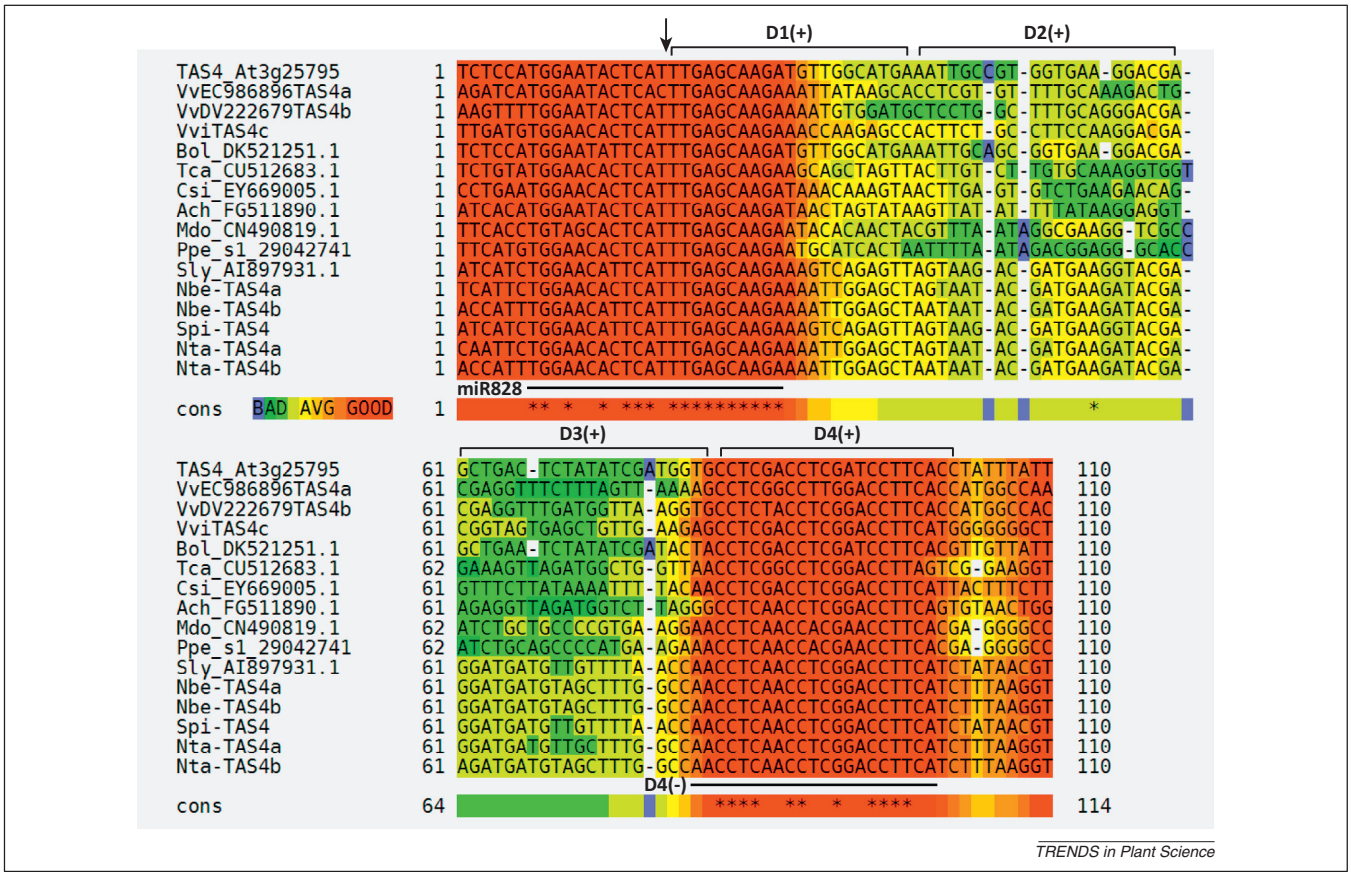

Figure 2. Evidence of selective sweep for expressed TAS4-3′D4, supporting *TAS4* function important for fitness of eudicots. Sequence alignment of *TAS4* paralogs revealed by BLASTing *TAS4/AT3G25795* (nt 870–980) or TAS4-3′-D4(–) in the GenBank plant EST database; IDs shown except *Prunus persica* = 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 Genome Survey Sequence (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 the 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 oleraca*; 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).

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 Figure S2C,D in the supplementary material online). 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* phasiRNAs, consistent with a higher binding affinity of TAS4b-3′D4(–) over that of TAS4a-3′D4(–) (see Figure S1B in the supplementary material online).

VvMYBA6 and VvMYBA7 functions in bioflavonoid synthesis

The novel *VvMYBA6, VvMYBA7*, 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 (*F1, PA1/2, VvMYBA1–3*) 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 (as are 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 paralogs, 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 has shown that homologs participate in the same processes [96]. The central positioning of VvMYBA6 and VvMYBA7 between S6 and S5/7/13 clades for anthocyanin and proanthocyanidin/flavonol/lignin 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 *VvMYBA1–3* isogenes clustered on Chr. 2 do not account for all observed

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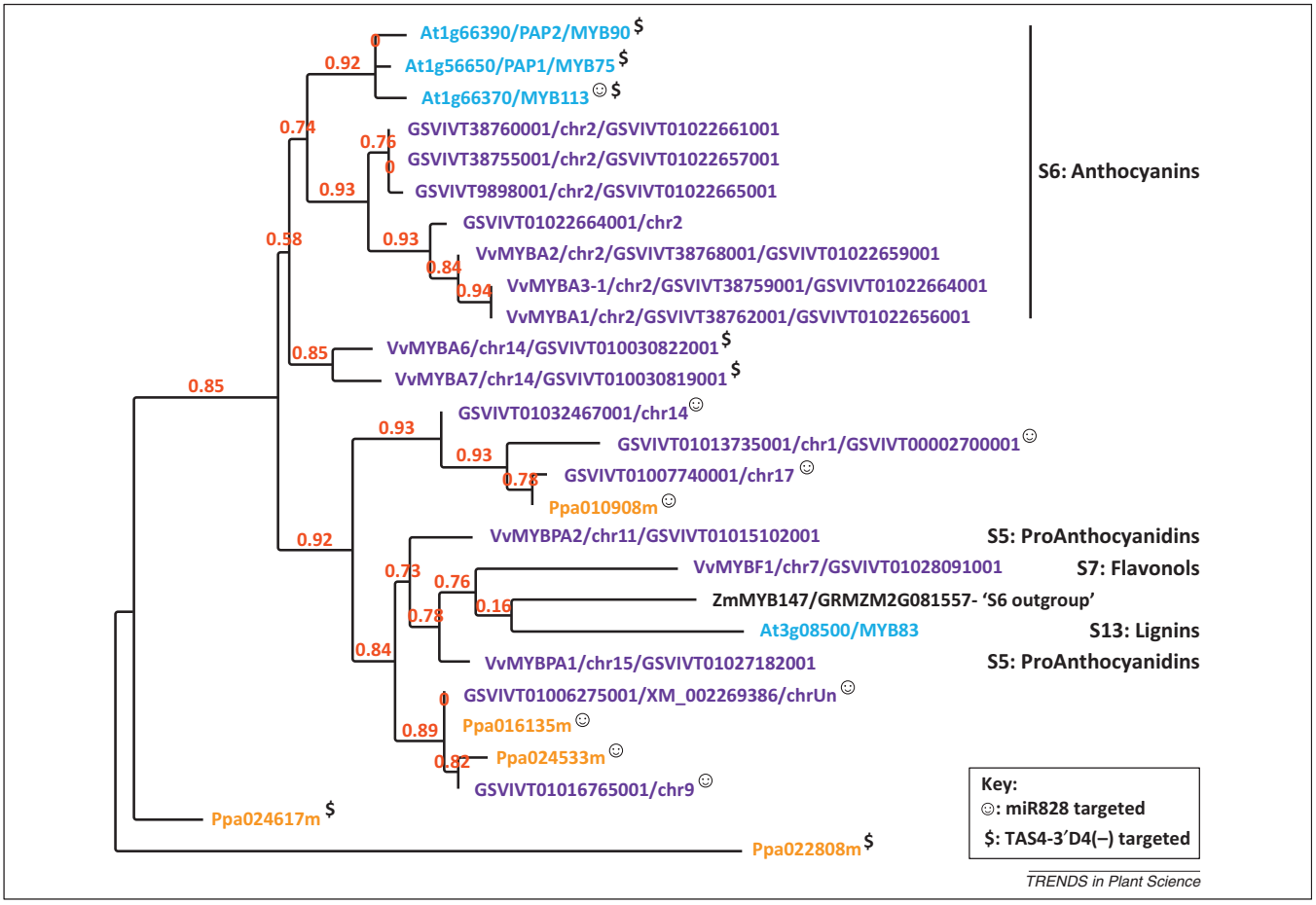

Figure 3. Phylogram of validated miR828 (☺)- and/or *TAS4*-targeted ($) grape (purple) or predicted peach [71] (orange) and *Arabidopsis* (blue) MYB proteins. Clustering of subgroups across species provides evidence of conserved MYB functions for synthesis of proanthocyanidins [subgroup 5 (S5)], anthocyanins (S6), and lignins (S13) based on functional analyses [96]. The subgroup functional annotations for maize (Zm prefix; outgroup gene most similar to S6), 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.

anthocyanin phenotypes [98] or flavonoid pathways [94], suggesting that VvMYBA6 and VvMYBA7 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 *VvMYBA1–3* isogenes in a Syrah×Grenache F_1 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 antidepressants Prozac and Paxil) early in the last century owing to their effects on the '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 phenylalanine (phe) catabolism by phenylalanine ammonia-lyase (*PAL*)]: flavonols by flavonol synthase (*FLS*) and UDP-glucuronic acid/galactose:flavonol-3-*O*-glucuronosyltransferases (*GT5/6*), anthocyanins

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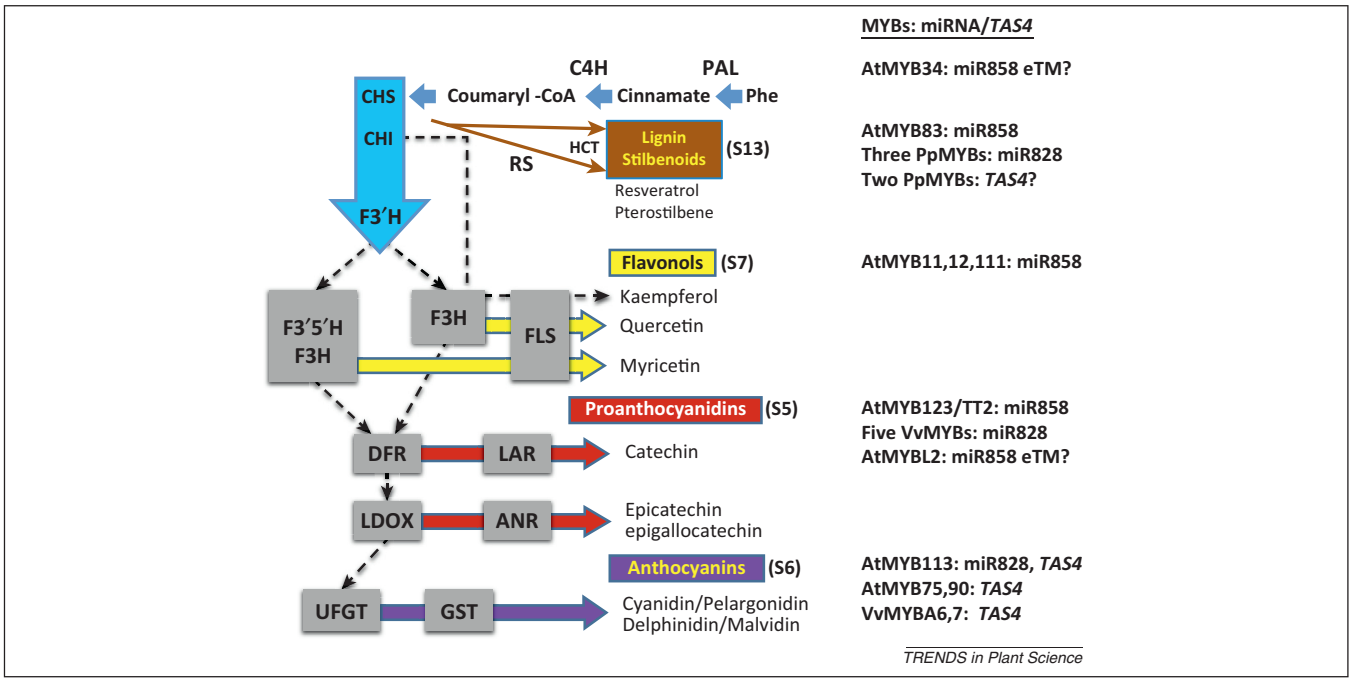

Figure 4. Simplified flavonoid biosynthetic pathway showing enzymatic steps specific to the biosynthesis of nutraceutical stilbenes (resveratrol synthase, RS), flavonols (flavonol synthase, FLS), proanthocyanidins (and lignin [110,111]) (hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase, HCT; leucoanthocyanidin reductase, LAR; and anthocyanidin reductase, ANR), and anthocyanins (UDP-glucose:flavonoid 3-*O*-glucosyltransferase, 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]. Other abbreviations: C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase; F3H/F3′R, flavanone 3- or 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; GST, gluthathione S-transferase; LDOX, leucoanthocyanidin dioxygenase; PAL, phenylalanine ammonia-lyase; Phe, phenylalanine; S5–S13, subgroups 5–13.

by UDP-glucose:flavonoid 3-*O*-glucosyltransferases (*UFGT*), 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 the evolution of *TAS4* and target *MYB*s

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 his 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, similarly to 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 upregulated 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

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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. Because *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 Vv*MYBA6/7* 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 leafroll-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 sRNA 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 together with *MYB*s 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 sRNA 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 the development of crops that respond efficiently 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 resulting opportunity to select for adaptability, for example for 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 the evolution of *MIR828*, *TAS4*, and *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 nucleotide-binding site–leucine-rich repeat (*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 National Institutes of Health (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

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did evolution adapt fermentation of flavonoid pathways that transform our preferred nutraceutical staples of cocoa and grape?

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2013.07.006.

References

1 Czech, B. and Hannon, G.J. (2011) Small RNA sorting: matchmaking for Argonautes. *Nat. Rev. Genet.* 12, 19–31
2 Carbonell, A. *et al.* (2012) Functional analysis of three *Arabidopsis* ARGONAUTES using Slicer-defective mutants. *Plant Cell* 24, 3613–3629
3 Allen, E. *et al.* (2005) microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. *Cell* 121, 207–221
4 Allen, E. and Howell, M.D. (2010) miRNAs in the biogenesis of *trans*-acting siRNAs in higher plants. *Semin. Cell Dev. Biol.* 21, 798–804
5 Rajeswaran, R. *et al.* (2012) Sequencing of RDR6-dependent double-stranded RNAs reveals novel features of plant siRNA biogenesis. *Nucleic Acids Res.* 40, 6241–6254
6 Rajagopalan, R. *et al.* (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev.* 20, 3407–3425
7 Garcia, D. *et al.* (2006) Specification of leaf polarity in *Arabidopsis* via the *trans*-acting siRNA pathway. *Curr. Biol.* 16, 933–938
8 MacLean, D. *et al.* (2010) Evidence for large complex networks of plant short silencing RNAs. *PLoS ONE* 5, e9901
9 Song, X. *et al.* (2012) Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. *Plant J.* 69, 462–474
10 Johnson, C. *et al.* (2009) Clusters and superclusters of phased small RNAs in the developing inflorescence of rice. *Genome Res.* 19, 1429–1440
11 Meng, Y.J. *et al.* (2013) Construction of gene regulatory networks mediated by vegetative and reproductive stage-specific small RNAs in rice (*Oryza sativa*). *New Phytol.* 197, 441–453
12 Hu, Z. *et al.* (2013) Analyses of a *Glycine max* degradome library identify microRNA targets and microRNAs that trigger secondary siRNA biogenesis. *J. Integr. Plant Biol.* 55, 160–176
13 Zhang, C. *et al.* (2012) Identification of *trans*-acting siRNAs and their regulatory cascades in grapevine. *Bioinformatics* 28, 2561–2568
14 Tang, S. *et al.* (2012) Identification of wounding and topping responsive small RNAs in tobacco (*Nicotiana tabacum*). *BMC Plant Biol.* 12, 15
15 Li, F. *et al.* (2012) SoMART: a web server for plant miRNA, tasiRNA and target gene analysis. *Plant J.* 70, 891–901
16 Kallman, T. *et al.* (2013) A significant fraction of 21 nt sRNA originates from phased degradation of resistance genes in several perennial species. *Plant Physiol.* 162, 741–754
17 Cuperus, J.T. *et al.* (2011) Evolution and functional diversification of *MIRNA* genes. *Plant Cell* 23, 431–442
18 Axtell, M.J. (2013) Classification and comparison of small RNAs from plants. *Annu. Rev. Plant Biol.* 64, 137–159
19 Montgomery, T.A. *et al.* (2008) Specificity of ARGONAUTE7-miR390 interaction and dual functionality in *TAS3 trans*-acting siRNA formation. *Cell* 133, 128–141
20 Zhang, X.L. *et al.* (2012) PUNCTATE VASCULAR EXPRESSION1 is a novel maize gene required for leaf pattern formation that functions downstream of the trans-acting small interfering RNA pathway. *Plant Physiol.* 159, 1453–1462
21 Zhai, J. *et al.* (2011) MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, *trans*-acting siRNAs. *Genes Dev.* 25, 2540–2553
22 Axtell, M.J. *et al.* (2006) A two-hit trigger for siRNA biogenesis in plants. *Cell* 127, 565–577
23 Cuperus, J.T. *et al.* (2010) Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in *Arabidopsis*. *Nat. Struct. Mol. Biol.* 17, 997–1003
24 Chen, H-M. *et al.* (2010) 22-nucleotide RNAs trigger secondary siRNA biogenesis in plants. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15269–15274
25 Manavella, P.A. *et al.* (2012) Plant secondary siRNA production determined by microRNA-duplex structure. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2461–2466
26 Yoshikawa, M. *et al.* (2013) 3′ fragment of miR173-programmed RISC-cleaved RNA is protected from degradation in a complex with RISC and SGS3. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4117–4122
27 Schott, G. *et al.* (2012) Differential effects of viral silencing suppressors on siRNA and miRNA loading support the existence of two distinct cellular pools of ARGONAUTE1. *EMBO J.* 31, 2553–2565
28 Hansen, T.B. *et al.* (2013) Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388
29 Wu, H-J. *et al.* (2013) Widespread long noncoding RNAs as endogenous target mimics for microRNAs in plants. *Plant Physiol.* 161, 1875–1884
30 Franco-Zorrilla, J.M. *et al.* (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat. Genet.* 39, 1033–1037
31 Meng, Y.J. *et al.* (2012) Target mimics: an embedded layer of microRNA-involved gene regulatory networks in plants. *BMC Genomics* 13, 12
32 Wang, K.C. and Chang, H.Y. (2012) Molecular mechanisms of long noncoding RNAs. *Mol. Cell* 43, 904–914
33 Lopez-Gomollon, S. *et al.* (2012) Diverse correlation patterns between microRNAs and their targets during tomato fruit development indicates different modes of microRNA actions. *Planta* 236, 1875–1887
34 Ivashuta, S. *et al.* (2011) Regulation of gene expression in plants through miRNA inactivation. *PLoS ONE* 6, e21330
35 Matsui, K. *et al.* (2008) AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in *Arabidopsis*. *Plant J.* 55, 954–967
36 Hsieh, L.C. *et al.* (2009) Uncovering small RNA-mediated responses to phosphate deficiency in *Arabidopsis* by deep sequencing. *Plant Physiol.* 151, 2120–2132
37 Luo, Q-J. *et al.* (2012) An autoregulatory feedback loop involving *PAP1* and *TAS4* in response to sugars in *Arabidopsis*. *Plant Mol. Biol.* 80, 117–129
38 Bhargava, A. *et al.* (2010) MYB75 functions in regulation of secondary cell wall formation in the *Arabidopsis* inflorescence stem. *Plant Physiol.* 154, 1428–1438
39 Borevitz, J.O. *et al.* (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* 12, 2383–2393
40 Jia, L. *et al.* (2012) Proanthocyanidins inhibit seed germination by maintaining a high level of abscisic acid in *Arabidopsis thaliana*. *J. Integr. Plant Biol.* 54, 663–673
41 Finkelstein, R.R. *et al.* (2002) Abscisic acid signaling in seeds and seedlings. *Plant Cell* 14, S15–S45
42 Riechmann, J.L. *et al.* (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science* 290, 2105–2110
43 Zhong, R. and Ye, Z-H. (2012) MYB46 and MYB83 bind to the SMRE sites and directly activate a suite of transcription factors and secondary wall biosynthetic genes. *Plant Cell Physiol.* 53, 368–380
44 Stracke, R. *et al.* (2010) Analysis of PRODUCTION OF FLAVONOL GLYCOSIDES-dependent flavonol glycoside accumulation in *Arabidopsis thaliana* plants reveals MYB11-, MYB12- and MYB111-independent flavonol glycoside accumulation. *New Phytol.* 188, 985–1000
45 Fahlgren, N. *et al.* (2007) High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of *MIRNA* genes. *PLoS ONE* 2, e219
46 Addo-Quaye, C. *et al.* (2008) Endogenous siRNA and miRNA targets identified by sequencing of the *Arabidopsis* degradome. *Curr. Biol.* 18, 758–762
47 Ciliberti, S. *et al.* (2007) Robustness can evolve gradually in complex regulatory gene networks with varying topology. *PLoS Comput. Biol.* 3, 164–173

Author's personal copy

48 Russo, P. *et al.* (2012) Flavonoids acting on DNA topoisomerases: recent advances and future perspectives in cancer therapy. *Curr. Med. Chem.* 19, 5287–5293
49 Spencer, J.P.E. (2010) The impact of fruit flavonoids on memory and cognition. *Br. J. Nutr.* 104, S40–S47
50 Jerison, H.J. (1973) *Evolution of the Brain and Intelligence,* Academic Press
51 Ratcliffe, J.M. (2009) Neuroecology and diet selection in phyllostomid bats. *Behav. Processes* 80, 247–251
52 Silcox, M.T. *et al.* (2009) Virtual endocast of *Ignacius graybullianus* (Paromomyidae, Primates) and brain evolution in early primates. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10987–10992
53 Barton, R.A. (1998) Visual specialization and brain evolution in primates. *Proc. R. Soc. Lond. Ser. B: Biol. Sci.* 26, 1933–1937
54 Alan, R.R. and McWilliams, S.R. (2013) Oxidative stress, circulating antioxidants, and dietary preferences in songbirds. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 164, 185–193
55 Jaillon, O. *et al.* (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature* 449, 463–467
56 Klevebring, D. *et al.* (2009) Genome-wide profiling of *Populus* small RNAs. *BMC Genomics* 10, 620
57 Pang, M. *et al.* (2009) Genome-wide analysis reveals rapid and dynamic changes in miRNA and siRNA sequence and expression during ovule and fiber development in allotetraploid cotton (*Gossypium hirsutum* L.). *Genome Biol.* 10, R122
58 Wang, F.D. *et al.* (2012) High-throughput sequencing discovery of conserved and novel microRNAs in Chinese cabbage (*Brassica rapa* L. ssp pekinensis). *Mol. Genet. Genomics* 287, 555–563
59 Barvkar, V. *et al.* (2013) Genome-wide identification and characterization of microRNA genes and their targets in flax (*Linum usitatissimum*). *Planta* 237, 1149–1161
60 Pelaez, P. *et al.* (2012) Identification and characterization of microRNAs in *Phaseolus vulgaris* by high-throughput sequencing. *BMC Genomics* 13, 83
61 Legrand, S. *et al.* (2010) One-step identification of conserved miRNAs, their targets, potential transcription factors and effector genes of complete secondary metabolism pathways after 454 pyrosequencing of calyx cDNAs from the Labiate *Salvia sclarea* L. *Gene* 450, 55–62
62 Garcia-Mas, J. *et al.* (2012) The genome of melon (*Cucumis melo* L.). *Proc. Natl. Acad. Sci. U.S.A.* 109, 11872–11877
63 Kim, J. *et al.* (2012) Small RNA and transcriptome deep sequencing proffers insight into floral gene regulation in Rosa cultivars. *BMC Genomics* 13, 657
64 Zuo, J.H. *et al.* (2012) Sculpting the maturation, softening and ethylene pathway: The influences of microRNAs on tomato fruits. *BMC Genomics* 13, 12
65 Bozorov, T. *et al.* (2012) Identification and profiling of miRNAs during herbivory reveals jasmonate-dependent and -independent patterns of accumulation in *Nicotiana attenuata*. *BMC Plant Biol.* 12, 209
66 Frazier, T. *et al.* (2010) Identification and characterization of microRNAs and their target genes in tobacco (*Nicotiana tabacum*). *Planta* 232, 1289–1308
67 Argout, X. *et al.* (2011) The genome of *Theobroma cacao*. *Nat. Genet.* 43, 101–108
68 Zhang, Q. *et al.* (2013) The genome of *Prunus mume*. *Nat. Commun.* 3, 1318
69 Gao, Z.H. *et al.* (2012) High-throughput sequencing of small RNAs and analysis of differentially expressed microRNAs associated with pistil development in Japanese apricot. *BMC Genomics* 13, 14
70 Eldem, V. *et al.* (2012) Genome-wide identification of miRNAs responsive to drought in peach (*Prunus persica*) by high-throughput deep sequencing. *PLoS ONE* 7, e50298
71 Zhu, H. *et al.* (2012) Unique expression, processing regulation, and regulatory network of peach (*Prunus persica*) miRNAs. *BMC Plant Biol.* 12, 18
72 Xu, Q. *et al.* (2012) The draft genome of sweet orange (*Citrus sinensis*). *Nat. Genet.* 45, 59–66
73 Xia, R. *et al.* (2012) Apple miRNAs and tasiRNAs with novel regulatory networks. *Genome Biol.* 13, R47
74 Wang, Z.J. *et al.* (2012) Discovery and profiling of novel and conserved microRNAs during flower development in *Carya cathayensis* via deep sequencing. *Planta* 236, 613–621
75 Patanun, O. *et al.* (2013) Computational identification of microRNAs and their targets in cassava (*Manihot esculenta* Crantz.). *Mol. Biotechnol.* 53, 257–269
76 Guzman, F. *et al.* (2012) Identification of microRNAs from *Eugenia uniflora* by high-throughput sequencing and bioinformatics analysis. *PLoS ONE* 7, e49811
77 Wang, K. *et al.* (2012) Identification of conserved and novel microRNAs from *Liriodendron chinense* floral tissues. *PLoS ONE* 7, e44696
78 Khraiwesh, B. *et al.* (2013) Identification and analysis of Red Sea Mangrove (*Avicennia marina*) microRNAs by high-throughput sequencing and their association with stress responses. *PLoS ONE* 8, e60774
79 Crane, P.R. *et al.* (1986) Lower Cretaceous angiosperm flowers: fossil evidence on early radiation of dicotyledons. *Science* 232, 852–854
80 Levey, D.J. *et al.*, eds (2002) *Seed Dispersal and Frugivory: Ecology, Evolution, and Conservation*, CABI Publications
81 Wan, L-C. *et al.* (2012) Identification and characterization of small non-coding RNAs from Chinese fir by high throughput sequencing. *BMC Plant Biol.* 12, 146
82 Hao, D.C. *et al.* (2012) Identification of *Taxus* microRNAs and their targets with high-throughput sequencing and degradome analysis. *Physiol. Plant* 146, 388–403
83 Yakovlev, I.A. *et al.* (2010) MicroRNAs, the epigenetic memory and climatic adaptation in Norway spruce. *New Phytol.* 187, 1154–1169
84 Lin, J-S. *et al.* (2012) MicroR828 regulates lignin and H_2O_2 accumulation in sweet potato on wounding. *New Phytol.* 196, 427–440
85 Wang, C. *et al.* (2012) Identification of microRNAs from Amur grape (*Vitis amurensis* Rupr.) by deep sequencing and analysis of microRNA variations with bioinformatics. *BMC Genomics* 13, 122
86 Pantaleo, V. *et al.* (2010) Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. *Plant J.* 62, 960–976
87 Fahlgren, N. and Carrington, J.C. (2010) miRNA target prediction in plants. In *Plant MicroRNAs* (Meyers, B.C. and Green, P.J., eds), pp. 51–57, Humana Press
88 Kuhn, C-D. and Joshua-Tor, L. (2013) Eukaryotic Argonautes come into focus. *Trends Biochem. Sci.* 38, 263–271
89 Luo, Q-J. *et al.* (2009) Evidence for antisense transcription associated with microRNA target mRNAs in *Arabidopsis*. *PLoS Genet.* 5, e1000457
90 Matus, J.T. *et al.* (2008) Analysis of the grape MYB R2R3 subfamily reveals expanded wine quality-related clades and conserved gene structure organization across *Vitis* and *Arabidopsis* genomes. *BMC Plant Biol.* 8, 83
91 Azuma, A. *et al.* (2011) Haplotype composition at the color locus is a major genetic determinant of skin color variation in *Vitis labruscana* grapes. *Theor. Appl. Genet.* 122, 1427–1438
92 Bogs, J. *et al.* (2007) The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. *Plant Physiol.* 143, 1347–1361
93 Cutanda-Perez, M-C. *et al.* (2009) Ectopic expression of VlmybA1 in grapevine activates a narrow set of genes involved in anthocyanin synthesis and transport. *Plant Mol. Biol.* 69, 633–648
94 Czemmel, S. *et al.* (2009) The grapevine R2R3-MYB transcription factor VvMYBF1 regulates flavonol synthesis in developing grape berries. *Plant Physiol.* 151, 1513–1530
95 Terrier, N. *et al.* (2009) Ectopic expression of VvMybPA2 promotes proanthocyanidin biosynthesis in grapevine and suggests additional targets in the pathway. *Plant Physiol.* 149, 1028–1041
96 Dubos, C. *et al.* (2010) MYB transcription factors in *Arabidopsis*. *Trends Plant Sci.* 15, 573–581
97 Du, H. *et al.* (2012) The R2R3-MYB transcription factor gene family in maize. *PLoS ONE* 7, e37463
98 Shimazaki, M. *et al.* (2011) Pink-colored grape berry is the result of short insertion in intron of color regulatory gene. *PLoS ONE* 6, e21308
99 Fournier-Level, A. *et al.* (2009) Quantitative genetic bases of anthocyanin variation in grape (*Vitis vinifera* L. ssp sativa) berry: a quantitative trait locus to quantitative trait nucleotide integrated study. *Genetics* 183, 1127–1139
100 Stenkamp, S. *et al.* (2009) Clonal variation and stability assay of chimeric Pinot Meunier (*Vitis vinifera* L.) and descending sports. *Euphytica* 165, 197–209

Author's personal copy

101 Lewis, D.R. *et al.* (2011) Auxin and ethylene induce flavonol accumulation through distinct transcriptional networks. *Plant Physiol.* 156, 144–164
102 Zhang, J. *et al.* (2009) Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. *Plant J.* 57, 171–183
103 Pourcel, L. *et al.* (2007) Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends Plant Sci.* 12, 29–36
104 Hichri, I.N. *et al.* (2011) Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *J. Exp. Bot.* 62, 2465–2483
105 Schaart, J.G. *et al.* (2013) Identification and characterization of MYB–bHLH–WD40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (Fragaria × ananassa) fruits. *New Phytol.* 197, 454–467
106 Gonzalez, A. *et al.* (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. *Plant J.* 53, 814–827
107 Ramsay, N.A. and Glover, B.J. (2005) MYB–bHLH–WD40 protein complex and the evolution of cellular diversity. *Trends Plant Sci.* 10, 63–70
108 Ono, E. *et al.* (2010) Functional differentiation of the glycosyltransferases that contribute to the chemical diversity of bioactive flavonol glycosides in grapevines (*Vitis vinifera*). *Plant Cell* 22, 2856–2871
109 Cohen, S.D. *et al.* (2012) Impact of diurnal temperature variation on grape berry development, proanthocyanidin accumulation, and the expression of flavonoid pathway genes. *J. Exp. Bot.* 63, 2655–2665
110 Zhao, Q. and Dixon, R.A. (2011) Transcriptional networks for lignin biosynthesis: more complex than we thought? *Trends Plant Sci.* 16, 227–233
111 Dardick, C. *et al.* (2010) Stone formation in peach fruit exhibits spatial coordination of the lignin and flavonoid pathways and similarity to *Arabidopsis* dehiscence. *BMC Biol.* 8, 13
112 Zheng, Y. *et al.* (2009) Sugars induce anthocyanin accumulation and flavanone 3-hydroxylase expression in grape berries. *Plant Growth Reg.* 58, 251–260
113 Matus, J.T. *et al.* (2009) Post-véraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*. *J. Exp. Bot.* 60, 853–867
114 Deluc, L.G. *et al.* (2011) Water deficit increases stilbene metabolism in Cabernet Sauvignon berries. *J. Agric. Food Chem.* 59, 289–297
115 Olle, D. *et al.* (2010) Effect of pre- and post-véraison water deficit on proanthocyanidin and anthocyanin accumulation during Shiraz berry development. *Aust. J. Grape Wine Res.* 17, 90–100
116 Friedman, W.E. (2009) The meaning of Darwin's 'abominable mystery'. *Am. J. Bot.* 96, 5–21
117 Staiger, D. *et al.* (2013) Emerging role for RNA-based regulation in plant immunity. *New Phytol.* 197, 394–404
118 Qiao, Y. *et al.* (2013) Oomycete pathogens encode RNA silencing suppressors. *Nat. Genet.* 45, 330–333
119 Campo, S. *et al.* (2013) Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the Nramp6 (Natural resistance-associated macrophage protein 6) gene involved in pathogen resistance. *New Phytol.* 199, 212–227
120 Wallis, C.M. and Chen, J. (2012) Grapevine phenolic compounds in xylem sap and tissues are significantly altered during infection by *Xylella fastidiosa*. *Phytopathology* 102, 816–826
121 De La Fuente, L. *et al.* (2013) The bacterial pathogen *Xylella fastidiosa* affects the leaf ionome of plant hosts during infection. *PLoS ONE* 8, e62945
122 Reuveni, M. and Reuveni, R. (1995) Efficacy of foliar application of phosphates in controlling powdery mildew fungus on field-grown winegrapes: effects on cluster yield and peroxidase activity in berries. *J. Phytopathol.* 143, 21–25
123 Skinner, P.W. and Matthews, M.A. (1990) A novel interaction of magnesium translocation with the supply of phosphorus to roots of grapevine (*Vitis vinifera* L.). *Plant Cell Environ.* 13, 821–826
124 Seo, M. and Koshiba, T. (2011) Transport of ABA from the site of biosynthesis to the site of action. *J. Plant Res.* 124, 501–507
125 Vega, A. *et al.* (2011) Compatible GLRaV-3 viral infections affect berry ripening decreasing sugar accumulation and anthocyanin biosynthesis in *Vitis vinifera*. *Plant Mol. Biol.* 77, 261–274
126 Pant, B.D. *et al.* (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant J.* 53, 731–738
127 Zhao, H. *et al.* (2013) Small RNA profiling reveals phosphorus deficiency as a contributing factor in symptom expression for citrus Huanglongbing disease. *Mol. Plant* 6, 301–310
128 Perea-Garcia, A. *et al.* (2013) *Arabidopsis* copper transport protein COPT2 participates in the cross talk between iron deficiency responses and low-phosphate signaling. *Plant Physiol.* 162, 180–194
129 Ding, Y. *et al.* (2013) Emerging roles of microRNAs in the mediation of drought stress response in plants. *J. Exp. Bot.* 64, 3077–3086
130 Shi, H. *et al.* (2013) *Arabidopsis* ALTERED MERISTEM PROGRAM 1 negatively modulates plant responses to abscisic acid and dehydration stress. *Plant Physiol. Biochem.* 67, 209–216
131 Shi, Y. *et al.* (2013) The glutamate carboxypeptidase AMP1 mediates abscisic acid and abiotic stress responses in *Arabidopsis*. *New Phytol.* 199, 135–150
132 Li, S.B. *et al.* (2013) MicroRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in *Arabidopsis*. *Cell* 153, 562–574
133 Jouannet, V. *et al.* (2012) Cytoplasmic *Arabidopsis* AGO7 accumulates in membrane-associated siRNA bodies and is required for ta-siRNA biogenesis. *EMBO J.* 31, 1704–1713
134 Stalder, L. *et al.* (2013) The rough endoplasmatic reticulum is a central nucleation site of siRNA-mediated RNA silencing. *EMBO J.* 32, 1115–1127
135 Brodersen, P. *et al.* (2012) Isoprenoid biosynthesis is required for miRNA function and affects membrane association of ARGONAUTE 1 in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1778–1783
136 Riaz, S. *et al.* (2008) Fine-scale genetic mapping of two Pierce's disease resistance loci and a major segregation distortion region on chromosome 14 of grape. *Theor. Appl. Genet.* 117, 671–681
137 Alabi, O.J. *et al.* (2012) High-throughput sequence analysis of small RNAs in grapevine (*Vitis vinifera* L.) affected by grapevine leafroll disease. *Mol. Plant Pathol.* 13, 1060–1076
138 Gutha, L. *et al.* (2010) Modulation of flavonoid biosynthetic pathway genes and anthocyanins due to virus infection in grapevine (*Vitis vinifera* L.) leaves. *BMC Plant Biol.* 10, 187
139 Bazin, J. *et al.* (2012) Complexity of miRNA-dependent regulation in root symbiosis. *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* 367, 1570–1579
140 Zuanazzi, J.A.S. *et al.* (1998) Production of *Sinorhizobium meliloti* nod gene activator and repressor flavonoids from *Medicago sativa* roots. *Mol. Plant Microbe Interact.* 11, 784–794
141 Shivaprasad, P.V. *et al.* (2012) A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. *Plant Cell* 24, 859–874
142 Turner, M. *et al.* (2012) Genome organization and characteristics of soybean microRNAs. *BMC Genomics* 13, 169
143 Li, F. *et al.* (2012) MicroRNA regulation of plant innate immune receptors. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1790–1795
144 Vaucheret, H. (2009) AGO1 homeostasis involves differential production of 21-nt and 22-nt miR168 species by *MIR168a* and *MIR168b*. *PLoS ONE* 4, e6442
145 Li, H. *et al.* (2010) Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation. *Plant Physiol.* 153, 1759–1770
146 Song, Q-X. *et al.* (2011) Identification of miRNAs and their target genes in developing soybean seeds by deep sequencing. *BMC Plant Biol.* 11, 5
147 Xie, Z. *et al.* (2003) Negative feedback regulation of Dicer-Like1 in *Arabidopsis* by microRNA-guided mRNA degradation. *Curr. Biol.* 13, 784–789
148 Morrell, P.L. *et al.* (2012) Crop genomics: advances and applications. *Nat. Rev. Genet.* 13, 85–96
149 Olsen, K.M. and Wendel, J.F. (2013) A bountiful harvest: genomic insights into crop domestication phenotypes. *Annu. Rev. Plant Biol.* 64, 47–70
150 Seo, J.K. *et al.* (2013) Contribution of small RNA pathway components in plant immunity. *Mol. Plant Microbe Interact.* 26, 617–625
151 Nystedt, B. *et al.* (2013) The Norway spruce genome sequence and conifer genome evolution. *Nature* 497, 579–584
152 Rigault, P. *et al.* (2011) A white spruce gene catalog for conifer genome analyses. *Plant Physiol.* 157, 14–28
153 Human Microbiome Project Consortium (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214