Transgenic Res (2020) 29:355–367
https://doi.org/10.1007/s11248-020-00196-w

BRIEF COMMUNICATION

CRISPR/Cas9-mediated targeted mutagenesis of *TAS4* and *MYBA7* loci in grapevine rootstock 101-14

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Received: 19 June 2019 / Accepted: 21 February 2020 / Published online: 23 April 2020

Abstract Pierce's disease (PD) of grapevine (*Vitis vinifera*) is caused by the bacterium *Xylella fastidiosa* and is vectored by xylem sap-sucking insects, whereas *Grapevine Red Blotch Virus* (GRBV) causes Red Blotch Disease and is transmitted in the laboratory by alfalfa leafhopper *Spissistilus festinus*. The significance of anthocyanin accumulations in distinct tissues of grapevine by these pathogens is unknown, but vector feeding preferences and olfactory cues from host anthocyanins may be important for these disease etiologies. Phosphate, sugar, and UV light are known to regulate anthocyanin accumulation via miR828 and *Trans-Acting Small-interfering locus4* (*TAS4*), specifically in grape by production of phased *TAS4a/b/c* small-interfering RNAs that are differentially expressed and target *MYBA5/6/7* transcription factor transcripts for post-transcriptional slicing and antisense-mediated silencing. To generate materials that can critically test these genes' functions in PD and GRBV disease symptoms, we produced transgenic grape plants targeting *TAS4b* and *MYBA7* using CRISPR/Cas9 technology. We obtained five *MYBA7* lines all with bi-allelic editing events and no off-targets detected at genomic loci with homology to the guide sequence. We obtained two independent edited *TAS4b* lines; one bi-allelic, the other heterozygous while both had fortuitous evidences of bi-allelic *TAS4a* off-target editing events at the paralogous locus. No visible anthocyanin accumulation phenotypes were observed in regenerated plants, possibly due to the presence of genetically redundant *TAS4c* and *MYBA5/6* loci or absence of inductive environmental stress conditions. The editing events encompass single base insertions and di/trinucleotide deletions of *Vvi-TAS4a/b* and *Vvi-MYBA7* at expected positions 3 nt upstream from the guideRNA proximal adjacent motifs NGG. We also identified evidences of homologous recombinations of *TAS4a* with *TAS4b* at the *TAS4a* off-target in one of the *TAS4b* lines, resulting in a chimeric locus with a bi-allelic polymorphism, supporting independent recombination events in transgenic plants associated with apparent high Cas9 activities. The lack of obvious visible pigment phenotypes in edited plants precluded pathogen challenge tests of the role of anthocyanins in host PD and GRBV resistance/tolerance mechanisms. Nonetheless, we demonstrate successful genome-editing of non-coding RNA and *MYB* transcription factor loci which can serve future characterizations of the functions of *TAS4a/b/c* and *MYBA7* in developmental, physiological, and environmental biotic/abiotic stress response pathways important for value-

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11248-020-00196-w) contains supplementary material, which is available to authorized users.

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added nutraceutical synthesis and pathogen responses of winegrape.

Keywords MYB transcription factor · microRNA · Flavonoids · Genome editing · Off-target editing · RNA interference · Genetically modified organisms

Background

Historically, plant diseases have been controlled by the application of chemical pesticides, commonly leading to residual contamination, negative impacts on beneficial insects, and vector insecticide resistance (Stelinski et al. 2012). Host pathogen resistance and crop quality improvements depends on applying new genetic insights and new technologies to accelerate breeding through improved genotyping and phenotyping methods, and by exploiting the available diversity in germplasm. The genetic identity of traditional grapevine (*Vitis vinifera*) cultivars used for wine discourages breeding approaches because markets and appellation statutes dictate cultivar choice, thus varieties lack recombination (Myles et al. 2011) and the resultant opportunity to select/screen for adaptability. Genome editing technologies, on the other hand, can result in non-"genetically modified organisms" (GMO) after outcrossing the effector transgene locus. Recently the USDA issued a directive that the agency does not have plans to regulate plants generated using gene editing techniques that create deletions/insertions that could otherwise have been developed through traditional breeding techniques (https://www.usda.gov/media/press-releases/2018/03/28/secretary-perdue-issues-usda-statement-plant-breeding-innovation), expanding prospects for genome editing of crops for resistance to insect pests and pathogens (Bisht et al. 2019; Mushtaq et al. 2019).

Two grapevine pathogens in particular [*Grapevine Red Blotch Virus* (GRBV) and *Xylella fastidiosa* (XF)] cause host disease symptoms that implicate anthocyanin as effectors that could mediate disease spreads in vineyards. There is evidence for host plant stress physiology associated with disease vector feeding deterrence in grapevine (Krugner et al. 2012). Some anthocyanin and derivative tannic compounds can reduce insect feeding (Johnson et al. 2010), including sap-sucking insects (Barbehenn and Constabel 2011; Makoi et al. 2010), which provides a plausible basis for observed XF infection susceptibility differences between anthocyanless and red cultivars (Cantos et al. 2002; Krivanek and Walker 2004; Raju and Goheen 1981). Prior work demonstrated that XF infection causes a significant decrease in leaf elemental phosphorus content of leaves (De La Fuente et al. 2013), and anthocyanin accumulation is a well-known plant physiological response to inorganic phosphate (P_i) starvation or sucrose treatment, including grapevine (Yamakawa et al. 1983; Yin et al. 2012). Based on their mobile nature in vascular tissues, P_i, sugars, the plant stress hormone abscisic acid (ABA), microRNAs (miRNAs), and target mRNAs have been recognized as systemic signals that convey the whole-plant P_i status internally (Lin et al. 2008, 2018; Thieme et al. 2015). Phytoalexin polyphenolics accumulate in xylem sap and leaves of XF-infected almonds (Wilhelm et al. 2011) and grape (Wallis and Chen 2012); some cultivars (e.g. 'Rubired') induce polyphenolics to higher concentrations and do not develop PD symptoms as quickly as anthocyanless cultivars such as 'Chardonnay' or 'Thompson Seedless' (Wallis et al. 2013). Phenolics inhibit XF growth in vitro (Maddox et al. 2010), and foliar applications of ABA increase xylem sap polyphenolics and promote curing of XF-infected grapevines in the greenhouse (Meyer and Kirkpatrick 2011).

Grapevine Red Blotch Virus (GRBV) is a monopartite, grapevine-infecting Grablovirus causing Red Blotch Disease and was first observed in California in 2008 (Calvi 2011). Bahder et al. (2016) identified the alfalfa leafhopper *Spissistilus festinus* as the candidate vector that can transmit GRBV under laboratory conditions. GRBV disease symptoms manifest as red patches due to anthocyanin accumulation in the middle of the grapevine leaf and in veins and petiole, which coalesce at the end of the growing season (Sudarshana et al. 2015). GRBV infection results in delayed and uneven berry ripening, higher titratable acids, reduced sugar and reduced anthocyanin content in the berry (Oberhoster et al. 2016), impairing fruit qualities which threaten both table grape and wine industries (Rwahnih et al. 2015).

XF is a gram-negative, xylem-limited bacterium associated with a large number of crop diseases (Kyrkou et al. 2018) including Pierce's disease of grape (PD), alfalfa dwarf, phony peach disease, plum

leaf scald, citrus variegated chlorosis, leaf scorches of coffee, almond, mulberry, blueberry, and most recently Olive quick decline syndrome in Italy (Almeida and Nunney 2015). PD is vectored by xylem sap-sucking insects, in particular the Glassy-Winged Sharpshooter (GWSS; *Homalodisca vitripennis*), an invasive species that caused an epidemic of PD in southern California in the 1990s, and by the endemic blue-green sharpshooter *Graphocephala atropunctata* in the Pacific northwest and northern California. Obvious PD symptoms are anthocyanin accumulation in leaves at the scorched periphery and shriveling of berries that impacts fruit quality and yield. The threat of a PD epidemic in northern California and the Pacific Northwest like in southern and central California in the 1880s, 1930s, 1970s, and 1990s remains real.

New tools and management strategies are needed to combat grapevine diseases. Despite years of focused efforts by microbiologists, entomologists, and plant physiologist/pathologists, the molecular mechanisms of PD or GRBV disease etiology are not understood (Kyrkou et al. 2018; Yepes et al. 2018). We hypothesize that *Trans-acting small-interfering RNA locus4 (TAS4)* (Rajagopalan et al. 2006) is a molecular determinant of GRBV and PD host susceptibility. *TAS4* generates a ~1 kb long non-coding RNA spawning 'phased' siRNAs (phasiRNAs) in 21 nt register due to processive activity of DICER-LIKE4 (DCL4) triggered by miR828. miR828 is a P_i- (Hsieh et al. 2009) and ABA-regulated (Luo et al. 2012) miRNA that directly and indirectly targets MYeloBlastosis viral oncogene-like (*MYB)* transcription factors (Rajagopalan et al. 2006) *PRODUCTION OF ANTHOCYANIN1/PAP1/MYB75/Vvi-MYBA6*, *PAP2/MYB90/Vvi-MYBA7*, and *MYB113/Vvi-MYBA5* (Sunitha et al. 2019). Grapevine has one *MIR828* and three functionally conserved *TAS4* loci (a–c) with implications for differential MYB cleavage activities (Rock 2013; Sunitha et al. 2019). Although genome editing of animal non-coding RNAs has been demonstrated and the method has high potential for engineering crops (Basak and Nithin 2015), only one report to date describes CRISPR editing of a plant non-coding RNA involved in tomato ripening (Li et al. 2018). We have applied Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) genome editing technology (Cong et al. 2013; Jinek et al. 2012) to disrupt grapevine *Vvi-TAS4a/b* and *Vvi-MYBA7* host genes to enable future critical assessments of candidate effectors of PD and GBRV etiology.

Materials and methods

Plasmid construction

We obtained binary plasmid p201N-Cas9 (Jacobs et al. 2015; Jacobs and Martin 2016) (www.addgene.org plasmid #59175) and generated recombinant vectors using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs) to genome edit the, *VviTAS4b*, and its target *VviMYBA6/7* loci (Table 1). Specifically, 20 bp guide sequences for *Tas4b*, and *MYBA6* and *MYBA7* were mined (Liu et al. 2017) to minimize off-target potential (Bae et al. 2014), comprising $G(N)_{19}$ synthetic guide (sgRNA) upstream from a protospacer adjacent motif NGG and distal scaffold sequence for Cas9 activity (Jinek et al. 2012).

Agrobacterium-mediated grapevine rootstock 101-14 transformation

Recombinant vectors electroporated into *Agrobacterium* EHA105 (plus empty vector control) were used to transform embryogenic callus derived from anthers of commercially relevant grapevine rootstock 101-14 (which produces requisite marker anthocyanins for phenotyping) as fee-for-service from the UC Davis Plant Transformation Facility (Tricoli et al. 2014). Regenerated plants were shipped under USDA permit APHIS-BRS# 17-342-101m.

Genomic Southern blot analysis of transgene events

Total DNA was extracted from frozen grapevine leaves of regenerated transgenic events as described (Lodhi et al. 1994) and quantified with a Nanodrop microvolume spectrophotometer (Thermo-Fisher). DNA samples (10 µg) from control empty vector and transgenic plants were digested with either *Bam*HI or *Hin*dIII restriction enzymes (New England Biolabs), electrophoresed in 1% agarose gels in 1 × Tris–borate–EDTA, and subjected to Southern blot analysis (Southern 1975).

Agarose gels were blotted onto positively charged Amersham Hybond-N+ nylon membrane (GE Healthcare Life Sciences, USA) using the capillary transfer (Thermoscientific, USA) and the membrane was UV-crosslinked (SpectroLinker XL-1500, Spectroline, Westbury NY). PCR amplified *nptII* and *cas9* coding sequences were gel purified and randomly labelled with [α-^{32}P]dCTP, 3000 Ci/mmol (Perkin Elmer, www.perkinelmer.com) and used as probes. Hybridization was performed at 65°C for 16–20 h. Post-hybridization washes were performed as follows: The hybridization solution was discarded, and the blots were washed at 65°C twice with 2X SSC/0.5% SDS and four times with 0.2X SSC/0.5% SDS. The radioactivity signals were scanned using a Personal Molecular Imager™ system (www.bio-rad.com).

Detection of CRISPR/Cas9-induced genome editing of target genes

a) Targeted amplicon sequencing

Characterization of genome editing events of target genes was by done by Amplicon-Ez targeted amplicon sequencing (Genewiz, South Plainfield, NJ). A 300 bp amplicon comprising the gRNA target region was PCR amplified using KAPA HiFi HotStart ReadyMix. Partial Illumina adapters were fused to the 5′ end of the gene specific PCR primers (PCR Primer sequences are listed in Suppl. Table 7). The PCR products were gel purified using Zymoclean gel DNA recovery kit and subjected to polyacrylamide gel electrophoresis-based genotyping (Zhu et al. 2014) and sequenced (Genewiz). The targeted amplicon sequencing was repeated in vegetatively propagated transgenic plant clones.

b) Genomic library sequencing and analysis

Genomic libraries were prepared using 200 ng of total DNA as input according to the instructions provided by TruSeq Nano DNA Sample Preparation kit for 550 bp insert size (Illumina®). Six DNA libraries (two empty vector, two TAS4b, and two MYBA7 transgenic plants) were constructed with eight bp dual-indexed adapters. The quality of each library was assessed using an Agilent High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer. Equi-molar concentrations of libraries were pooled and sequenced on Illumina NovaSeq SP lane at the University of California, Los Angeles Genomics Core Facility. The reads obtained were analyzed using Magic-Blast (Boratyn et al. 2019) for targeted and off-target editing, SPAdes (Nurk et al. 2013) for de novo assembly of T-DNA integration scaffolds, and bowtie (Langmead et al. 2009) for non-T-DNA integration.

Results

Genome editing by CRISPR/Cas9 of *Vvi-TAS4b* and *Vvi-MYBA7* genes

The synthetic guide RNA (sgRNA) sequences of interest (Table 1), including potential off target loci, were identified by manual inspection and computationally (Bae et al. 2014; Liu et al. 2017). Synthetic oligonucleotides were designed to overlap with the U6 promoter sequence in p201N-cas9 as described (Jacobs et al. 2015; Jacobs and Martin 2016) to yield p201N-gRNA-cas9 using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). The p201N-cas9 vector harbors the neomycin

Table 1 Synthetic guide sequences for CRISPR-Cas9 editing of *VviMYBA6*, *MYBA7*, and *TAS4b* genes

Gene.test	Candidate guide sequence	Relative genome position	Off targets, seed (12)NGG? (seed mismatches, microhomology score; locus)
VviMYBA6.1	GGCCCTTCAGGAGTGCGGAA	Exon1, codon3, sense	No
VviMYBA7.1	GGCTCTTTAGGTCTGCGGAA	Exon1, codon3, sense	chr7:14830652 (2 mm, 0.4; intergenic)
VviTAS4b.2	CGGACCTTCACCATGGCCAC	D4 phase, sense	chr14:21607930rc (1 mm, 1.5; *TAS4a*)

Prioritized candidates were chosen based on dearth of canonical off targets with low seed microhomology scores (Bae et al. 2014; Liu et al. 2017)

phosphotransferaseII (*nptII*) gene as the plant selectable marker (Fig. 1a). We used *Agrobacterium* strain EHA105, a T-DNA deletion derivative of hypervirulent Ti plasmid pTiBo542 (Hood et al. 1993), carrying binary p201N-gRNA-cas9 vectors (Jacobs et al. 2015) targeting the *MIR828*, *TAS4a/b*, and *MYBA6* and *MYBA7* loci to transform embryogenic callus derived from anthers of the commercially relevant grapevine rootstock 101-14 (Tricoli et al. 2014). 101-14 produces requisite marker anthocyanins for phenotyping of transgene events. Grape transformation with p201N-gRNA-cas9 constructs listed in Table I yielded two kanamycin-resistant *TAS4b* plants (*TAS4b-1*, *TAS4b-2*), six *MYBA6* plants (*MYBA6*-1, -2, -3, -4, -6 and -7) and six *MYBA7* plants (*MYBA7*-1, -2, -3, -5, -6 and -8). We also obtained two empty vector (with no gRNA cassette) transgenic plants (cas9-1, cas9-2).

T-DNA integrations in kanamycin-resistant regenerated plants (expressing the *nptII* gene) were characterized by genomic Southern blots hybridized with *nptII* and *cas9* probes. Digestion of genomic DNA with *Hin*dIII and hybridization with the *nptII* probe is predicted to yield junction fragments for integration events that include the left T-DNA border outside of the selectable marker larger than 4.3 kb. The integration of the *cas9* sequence mapping inside the right T-DNA border was assayed by digestion of genomic DNA with *Bam*HI enzyme. Junction fragments larger than 2.7 kb are expected to hybridize when T-DNA

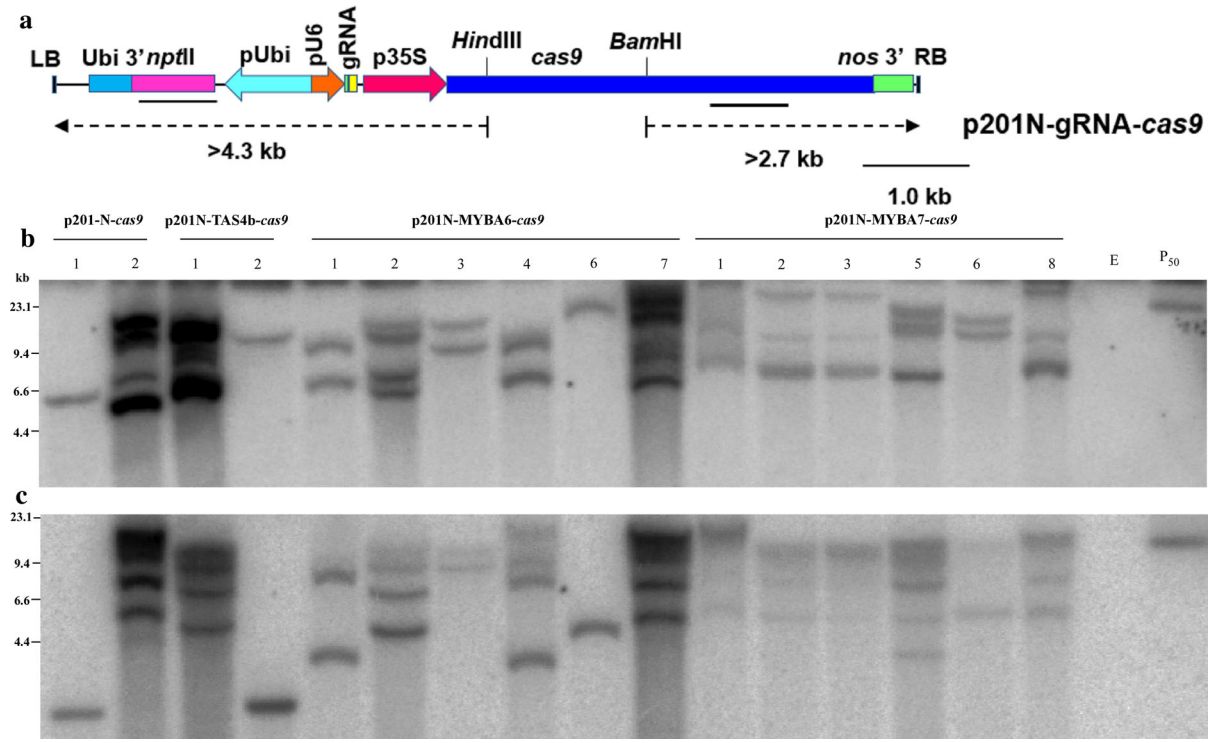


Fig. 1 Southern blot analysis of grape plants transformed with CRISPR vectors p201-gRNA-*cas9*. **a** The T-DNA of the binary vector p201N-gRNA-cas9. RB: T-DNA right border. p35S: Cauliflower mosaic virus 35S promoter. *cas9*: CRISPR associated protein9, human codon optimized. *nos 3′*: polyadenylation signal of the nopaline synthase gene. pU6: *Medicago truncatula* U6.6 promoter. gRNA: guide RNA. pUbi: maize ubiquitin promoter. *nptII*: neomycin phosphotransferase gene. Ubi 3′: ubiquitin 3′ polyadenylation signal. LB: T-DNA left border. Probes used (*nptII* and *cas9*) have been marked in bold lines. The junction fragment sizes > 4.3 kb and > 2.7 kb have been marked in a dashed arrow. **b**, **c** Southern blot analysis of grape plants transformed and regenerated with p201N-gRNA-*cas9* probed with *nptII* and *cas9*, respectively; P_{50} plasmid p201N-MYBA6-cas9 was used as hybridization positive control. Total DNA was extracted from 16 plants (two vector alone, two p201N-TAS4b-cas9, six p201N-MYBA6-*cas9*, and six p201N-MYBA7-*cas9*) rooted under kanamycin selection. **b** DNA (10 µg) digested with *Hin*dIII probed with *nptII*. T-DNA junction restriction fragments > 4.3 kb for transgenic plants are expected. **c** DNA (10 µg) digested with *Bam*HI probed with *cas9*. T-DNA junction restriction fragments > 2.7 kb for transgenic plants are expected. (Color figure online)

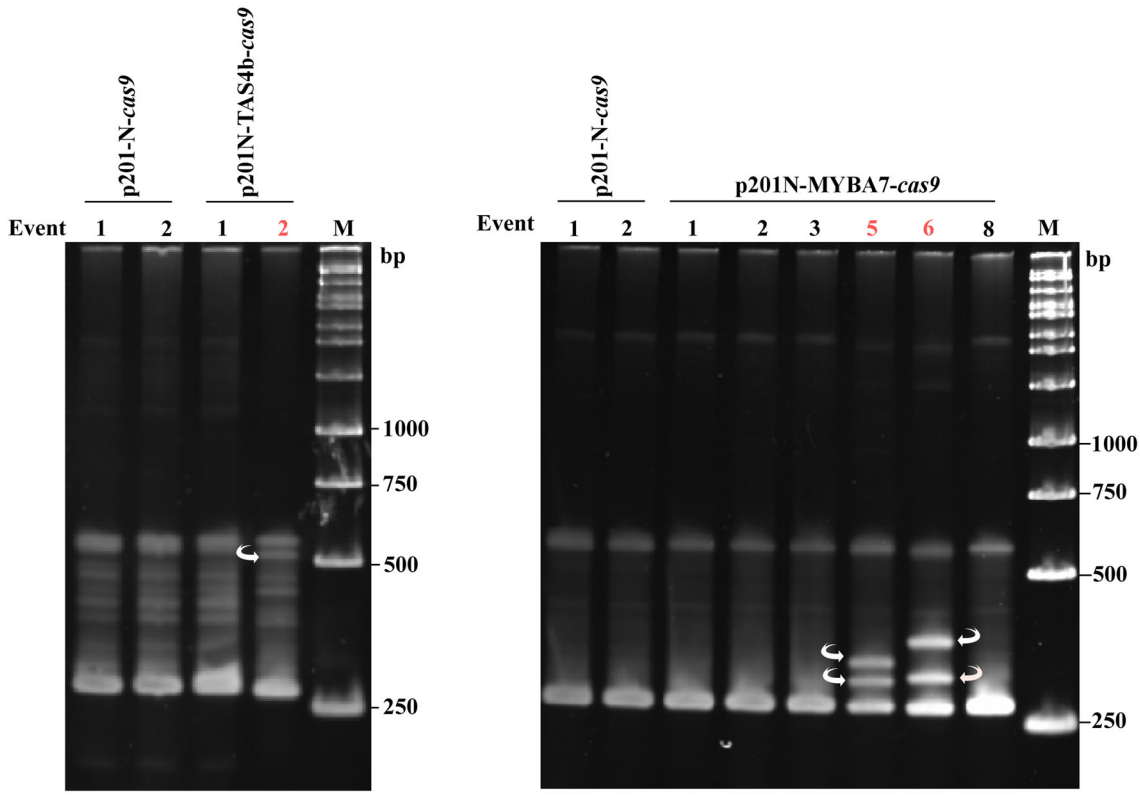
integration events include the right T-DNA border. Southern blot analysis showed that all of the transgenic plants had at least one integrated copy of the T-DNA harboring both the *nptII* (Fig. 1b) and *cas9* genes (Fig. 1c). Junction fragment analysis revealed that MYBA7-2 and -3 events were likely clones that regenerated from the same transformation event (Fig. 1b, c).

To initially identify candidate genome-edited events in transgenic plants, facile polyacrylamide gel electrophoresis-based genotyping (Zhu et al. 2014) was performed. PAGE heteroduplex analysis is based on the rationale that DNA heteroduplexes with bulges migrate in gels at a slower rate than homoduplexes. PCR amplification of target sequences results in a mixture of amplicons from template variants that can include edited allele(s). Denaturation and renaturation of PCR products result in homoduplexes if there is no template complexity or heteroduplexes with different gel migration rates. Based on the differences in migration of bands compared to vector alone regenerant control plants, Fig. 2a shows evidence for one candidate editing event for TAS4b (lane event number 2), and at least two editing events for MYBA7 (lane event numbers 5 and 6).

A 300 bp gRNA target region was amplified by PCR from genomic DNA template extracted from each transgenic line and vector-alone control line using primers containing partial Illumina adapter sequences incorporated in the 5′ end of the primers. The PCR products were gel-purified and assessed by targeted deep sequencing (Amplicon-EZ, Genewiz; South Plainfield, NJ) (Fig. 2b). The identified two bp editing deletion of MYBA7-5 (m5-1) changed the reading frame of the polypeptide while the in-frame three bp deletions for characterized MYBA7 line #6 (m6-1) at the target site are predicted to delete residue 8^{Arg} from the polypeptide, whereas the bi-allelic A/G single bp insertion dual mutations in MYBA7 lines #1, 2, 5, 6, and 8 are predicted to cause translation termination after residue 13^{Asp}. We also observed evidence of mono- and bi-allelic cas9-mediated events: a single bp insertion in TAS4b line #1 (t1-1) and 3 bp deletion in TAS4b #2 (t2-1). The editing events characterized by amplicon deep sequencing were independently confirmed by whole genome resequencing of libraries made from genomic DNAs extracted from two empty vector transgenic plants (cas9-1 and -2), two TAS4b edited plants (TAS4b-1 and -2), and two MYBA7 edited plants (MYBA7-5 and -6). We confirmed the accuracy of results from two rounds of amplicon deep sequencing by mapping whole genome resequencing datasets using Magic-Blast (Boratyn et al. 2019) (Suppl. Table 1). Empty vector-transformed transgenic plants had intact unedited target sites, confirmed by 12 TAS4b unedited reads and 27 MYBA7 reads, respectively. The editing event in TAS4b-1 (t1-1) was confirmed to be heterozygous (**+/-**) with six edited reads and 3 unedited reads found in whole genome resequencing libraries (Fig. 2b; Suppl. Table 1) while the three bp deletion of TAS4b-2 was confirmed to be bi-allelic (+/+) with 3 edited reads and zero unedited reads (Fig. 2b; Suppl. Table 1). Resequencing of the MYBA7-5 transgenic line was confirmed to be bi-allelic with a two bp deletion event m5-1 (2 reads) and a single bp insertion of "A" for m5-2 (7 reads). Similarly, MYBA7-6 was confirmed to be bi-allelic with a three bp deletion m6-1 (4 reads) and a single bp insertion of "A" for m6-2 (6 reads) (Fig. 2b; Suppl. Table 1). We did not detect any unedited wild type

Fig. 2 Evidence for genome editing of *TAS4b* and *MYBA7* in transgenic grapevine events. **a** Polyacrylamide gel electrophoresis heteroduplex amplicon assay (Zhu et al. 2014) showing candidate editing events in stably transformed grapevine regenerants (white arrows). p201-N-cas9: empty vector control regenerants. **b** Validation by deep sequencing of amplicons and genomic sequencing of transgenics for independent grapevine *TAS4b* and *MYBA7* CRISPR editing events (far right column) resulting in one nt insertions (bold italicized red), two nt or three nt deletions (bold dashes) at expected positions three nt upstream of the Proximal Adjacent Motif (PAM; underlined blue) in target guide sequences (italicized red). The *MYBA7* insertion events result in a stop codon five amino acids downstream from frame shifts. ***TAS4b-RC***: Reverse complement sequence of guide RNA; ***EV-1&2***: Empty vector transgenic plants cas9-1 and cas9-2; ***t1-1***: TAS4b-1edited event; ***t2-1***: TAS4b-2 edited event; ***m1-1* and *m1-2***: MYBA7-1 edited event 1 and 2; ***m2-1* and *m2-2***: MYBA7-2 edited event 1 and 2; ***m5-1* and *m5-2***: MYBA7-5 edited event 1 and 2; ***m6-1* and *m6-2***: MYBA7-6 edited event 1 and 2; ***m8-1* and *m8-2***: MYBA7-8 edited event 1 and 2. **c** Detection of off-target effect on TAS4a locus by genomic sequencing of TAS4b transgenic plants. TAS4a locus is unedited in empty vector transgenic plants (EV-1 and EV2) and in MYBA7 transgenic plants (MYBA7-5 and MYBA7-6). ***a1-1***: TAS4a locus edited TAS4b-1 plant; ***a2-1***, ***a2-2 and a2-3***: Three different editing events of TAS4a locus in TAS4b-2 plant. Color scheme: one nt insertion (bold italicized red); target sequences (italicized red); TAS4a/b recombinant sequences (italicized green underlined). **b**, **c** **-/-**: unedited genotype; **+/-**: mono-allelic editing; **+/+**: bi-allelic editing. (Color figure online)

a



b

Target Sequence			Amplicon Seq. reads/total	(%)	Event	Genotype	Resequencing Reads Edited/Unedited
TAS4b-chr14:21534645-21534686							
TAS4b-RC	5'..TGATTGGTGGCCATGGTGAAGGT_CCGAGGTAGAGGCACCTTA..3'	WT	179288/192572	(93.1%)	EV-1&2	-/-	0/12
t1-1	5'..TGATTGGTGGCCATGGTGAAGGTTCCGAGGTAGAGGCACCTTA..3'	+1	36480/74498	(48.9%)	TAS4b-1	+/-	6/3
t2-1	5'..TGATTGGTGGCCATGGTGAA---_CCGAGGTAGAGGCACCTTA..3'	-3	67984/72165	(94.2%)	TAS4b-2	+/+	3/0
MYBA7-chr14:16642727-16642769							
MYBA7	5'..ATGGAGGGCTCTTTAGGTCTGCG_GAAAGGTGCTTGGACTAGTG..3'	WT	171102/179149	(95.5%)	EV-1&2	-/-	0/27
m1-1	5'..ATGGAGGGCTCTTTAGGTCTGCGAGAAAGGTGCTTGGACTAGTG..3'	+1	28878/67504	(42.7%)	MYBA7-1	+/+	
m1-2	5'..ATGGAGGGCTCTTTAGGTCTGCGGGAAAGGTGCTTGGACTAGTG..3'	+1	36897/67504	(54.6%)	MYBA7-1		
m2-1	5'..ATGGAGGGCTCTTTAGGTCTGCGAGAAAGGTGCTTGGACTAGTG..3'	+1	38280/88298	(43.3%)	MYBA7-2	+/+	
m2-2	5'..ATGGAGGGCTCTTTAGGTCTGCGGGAAAGGTGCTTGGACTAGTG..3'	+1	47759/88298	(54.0%)	MYBA7-2		
m5-1	5'..ATGGAGGGCTCTTTAGGTCTG--_GAAAGGTGCTTGGACTAGTG..3'	-2	53312/93758	(56.9%)	MYBA7-5	+/+	2/0
m5-2	5'..ATGGAGGGCTCTTTAGGTCTGCGAGAAAGGTGCTTGGACTAGTG..3'	+1	38338/93758	(40.9%)	MYBA7-5		7/0
m6-1	5'..ATGGAGGGCTCTTTAGGTCT---_GAAAGGTGCTTGGACTAGTG..3'	-3	38280/88298	(42.9%)	MYBA7-6	+/+	4/0
m6-2	5'..ATGGAGGGCTCTTTAGGTCTGCGAGAAAGGTGCTTGGACTAGTG..3'	+1	47759/88298	(55.1%)	MYBA7-6		6/0
m8-1	5'..ATGGAGGGCTCTTTAGGTCTGCGAGAAAGGTGCTTGGACTAGTG..3'	+1	32101/75249	(42.7%)	MYBA7-8	+/+	
m8-2	5'..ATGGAGGGCTCTTTAGGTCTGCGGGAAAGGTGCTTGGACTAGTG..3'	+1	40737/75249	(54.1%)	MYBA7-8		

c

Off-target Sequence			Resequencing Reads Edited/Unedited	Genotype
TAS4a chr14:21607911-21607955-RC		Event		
	5'..CCACATTTTGGCCATGGTGAAGGT_CCAAGGCCGAGGCTTTTAA..3'	EV-1	0/16	-/-
	5'..CCACATTTTGGCCATGGTGAAGGT_CCAAGGCCGAGGCTTTTAA..3'	EV-2	0/8	-/-
	5'..CCACATTTTGGCCATGGTGAAGGT_CCAAGGCCGAGGCTTTTAA..3'	MYBA7-5	0/2	-/-
	5'..CCACATTTTGGCCATGGTGAAGGT_CCAAGGCCGAGGCTTTTAA..3'	MYBA7-6	0/9	-/-
a1-1	5'..CCACATTTTGGCCATGGTGAAGGTTCCAAGGCCGAGGCTTTTAA..3'	TAS4b-1	3/0	+/+
a2-1	5'..CCACATTTTGGCCATGGTGAAGGTTCCAAGGCCGAGGCTTTTAA..3'	TAS4b-2	5/0	
a2-2	5'..CCACATTTTGGCCATGGTGAAGGTTCCGAGGTAGAGGCACCTTA..3'	TAS4b-2	3/0	+/+
a2-3	5'..CCACATTTTGGCCATGGTGAAGGTTCCGAGGTCGAGGCACCTTA..3'	TAS4b-2	3/0	
	5'...TGATTGGTGGCCATGGTGAAGGT_CCGAGGTAGAGGCACCTTA..3'	TAS4b-chr14:21534645-21534686		

reads in MYBA7-5 and MYBA7-6. Presumably because of genetic redundancy of *TAS4* (three loci; a, b, c) and *MYBA7* (two paralogous loci, *MYBA5* and *MYBA6*), anthocyanin phenotypes did not obviously manifest in the regenerated transgenic lines.

Detection of an off-target effect from TAS4b- but not MYBA7-gRNAs

The predicted off-targets of TAS4b and MYBA7 gRNA were mined using the online tool CRISPR-P 2.0 (Liu et al. 2017) based on seed microhomology (Bae et al. 2014) and listed in Suppl. Table 2. Interestingly, we found polymorphism of the off-target sequence in 101-14 rootstocks when we analyzed resequencing data available in the public domain (Liang et al. 2019) (SRR5891889_1.fastq and SRR5891889_2.fastq), in comparison to the reference genome. The 101-14 polymorphic sequence of the off-target sequence is listed in Suppl. Table 2. We checked for off-target editing in the genomic resequencing data of two empty vector transgenic plants (cas9-1 and -2), two TAS4b edited plants (TAS4b-1 and -2) and two MYBA7 edited plants (MYBA7-5 and -6). The top off-target hit for *TAS4b* was its closest homolog *TAS4a* with two bp mismatch between the gRNA and the off-target sequence (Suppl. Table 2). The unedited off-target sequence was observed as wild type in empty vector (EV-1 and EV-2) and MYBA7 transgenic plants (MYBA7-5 and -6) (Fig. 2c; Suppl. Table 3). In the TAS4b-1 edited plant, we found the bi-allelic editing of off-target *TAS4a* sequence with a single bp insertion (a1-1) (Fig. 2c; Suppl. Table 3). In TAS4b-2 three bp bi-allelic deletion plant, we found two editing events in the *TAS4a* off-target locus. A single bp insertion at *TAS4a*, similar to the off-target event in TAS4b-1 plant was observed in TAS4b-2 plant (a2-1) (Fig. 2c; Suppl. Table 3). The second off target single base insertion editing event was coupled with apparent homologous recombinations between *TAS4a* and *TAS4b*. The most parsimonious account of the reads suggest Cas9-mediated dsDNA breaks in *TAS4a* resulted in independent single bp insertions three bp upstream of the PAM sequence followed by strand exchange with either intra- or inter-strand versions of the *TAS4b* locus (a2-2 and a2-3) (Fig. 2c; Suppl, Table 3). The evidence for independent recombination events are multiple reads in the same TAS4b-2 plant library of a C/A polymorphism found two nt downstream of the PAM in *TAS4a*. Consistent with independent homologous recombination events associated with high Cas9 guide activity is that no reciprocal exchange events with *TAS4b* (i.e. the complementary product of a single bi-molecular recombination reaction) were detected in the TAS4b-2 resequencing library. No off-target effects were observed in MYBA7 edited plants MYBA7-5 or -6, including for candidate off target MYBA6, the closest homolog of MYBA7 (Suppl. Table 4).

Characterization of T-DNA integration loci in transgenic plants

T-DNA integration is often incomplete with truncated T-DNA transfer (Spielmann and Simpson 1986; Yin and Wang 2000) and non-T-DNA vector backbone portions integrated randomly in the genome (Ramanathan and Veluthambi 1995). To check for non-T-DNA portion integrations we performed bowtie (Langmead et al. 2009) mapping of the resequencing libraries against the p201-N-cas9 vector sequence. The output revealed long T-DNA integrations past the left border in all transgenic plants assayed (Suppl. Table 5). We observed concordant increases in non-T-DNA integrations with increased copy numbers of T-DNA integrated evidenced by intense Southern hybridization signals in certain events (Fig. 1b; p201-N-cas9 empty vector event 2; TAS4b event 1). Next, we mapped the T-DNA integration loci by finding chimaeric 101 bp resequencing reads mapping to the vector yet having perfect homology at read end overhangs to the grapevine reference genome using Magic-Blast (Boratyn et al. 2019). We independently assessed the T-DNA integration event structures using de novo assembler SPAdes (Nurk et al. 2013). Both tools mapped at least one edge of an integration with evidence of chromosomal grape sequences chimaeric with a T-DNA left and or right border sequence. Specifically, we identified integration of the empty vector Cas9-1 event to chr8:2688372-2688357, MYBA7-6 event to chr15: 12878391–12878411 (Suppl. Table 6; Suppl.docx 1; Suppl.docx 2). Magic-Blast mapped TAS4b- 1 to chr12: 14666332–14666303 and TAS4b-2 to chr4: 10021502–10021519. We were unsuccessful by these methods to map the integration sites of either cas9-2 or MYBA7-5, most likely due to the complex, apparently higher copy number T-DNA integrations of these lines

(Fig. 1b). A further technical issue for these samples was the read depths of these two libraries were relatively shallow, ~ 78% and 37% respectively versus the average depths of the libraries successful to identify multiple chimaeric reads that established T-DNA integration loci (Suppl. Table 6). The consequence was the number of de novo-assembled contigs were higher for these two libraries, with corresponding shorter contig lengths.

Discussion

Many labs have demonstrated high efficiency (~ 80% including bi-allelic/homozygous mutations in primary transformants) of plant genome editing by CRISPR/Cas9 synthetic guide technologies, reviewed in Belhaj et al. (2013), Bisht et al. (2019), Mushtaq et al. (2019) and Raitskin and Patron (2016). CRISPR induces DNA double-strand breaks at specific genome sites that have a high propensity to result in multiple independent site-directed mutations through error-prone non-homologous end joining. Recent results including deployment of CRISPR-Cas12a (Cpf1) from *Prevotella* and *Francisella* show the method is practical for engineering resistance in Duncan grapefruit to citrus canker (Jia et al. 2015, 2019) caused by the bacterium *Xanthomonas axonopodis* by modifying the PthA4 pathogenicity effector binding elements in the promoter of *Cs-Lateral Organ Boundaries1* susceptibility gene. Five reports have documented the efficacy of creating events by CRISPR/Cas9 *Agrobacterium*-mediated transformation/regeneration in grapevine: targeting *Vvi-Phytoene Desaturase* in cv Muscat (Nakajima et al. 2017) and cv Chardonnay and 41B (Ren et al. 2019) as visible marker for bi-allele knockout efficiency, the *Vvi-WRKY52* gene (~ 70% biallelic events) in cv Thompson seedless for resistance to noble rot caused by *Botrytis cinerea* (Wang et al. 2018), and the *Vvi-L-idonate dehydrogenase/IdnDH* gene for tartaric acid biosynthesis in cv Chardonnay (Osakabe et al. 2018; Ren et al. 2016). In each case, and especially the latter, a preponderance of events were one bp insertions and three bp deletions like we observed for predominantly bi-allelic *Vvi-TAS4ab* and *Vvi-MYBA7* regenerants (Fig. 2). Another report documented grapevine cv Chardonnay protoplasts as suitable starting material for CRISPR/Cas9 editing at an estimated rate of 0.1% indels generated in a candidate powdery mildew susceptibility gene *MLO-7* (Malnoy et al. 2016). Because transgenic cells expressing CRISPR/Cas9 constructs are subject to mutations arising independently as a function of Cas9 activity, the limitations of low transformation efficiency in grapevine appear to have been overcome by high Cas9 performance in our transgene events (Fig. 2b, c). Nakajima et al. (2017) observed from the visible phenotype of bi-allelic CRISPR-induced mutations in the *Phytoene Desaturase1* gene of grapevine a correlation between leaf age and mutation rates.

Target specificity is an important issue for all genome editing technologies, including CRISPR/Cas9. Off-targets have been addressed with experimental evidence for some off-target activity in rice, barley and *Brassica oleracea* (Lawrenson et al. 2015) but not in *N. benthamiana* [reviewed in Raitskin and Patron (2016)] or in the documented cases of grapevine CRISPR (Nakajima et al. 2017; Osakabe et al. 2018; Ren et al. 2016, 2019; Wang et al. 2018). We observed fortuitous off-target mutations of *TAS4b* homolog *TAS4a*, wherein the off-target sequence had just two bp mismatches between the *TAS4b* gRNA and the off target. We observed a single bp insertion in TAS4b-1 and -2 plants and novel homologous recombinations of *TAS4a-TAS4b* loci in the TAS4b-2 plant remarkably with apparent breakpoints at the Cas9-induced dsDNA break (Fig. 2c). Several recent works have shown CRISPR-mediated targeted recombination in Drosophila (Brunner et al. 2019), yeast (Sadhu et al. 2016), and tomato (Filler-Hayut et al. 2017). Studies have shown that design of gRNAs with at least three mismatches from other genomic regions can alleviate off-target editing (Young et al. 2019). Polymorphism of *TAS4a* locus in 101-14 rootstock genotype appears to reduce the number of mismatches to two residues compared to the Pinot Noir reference genome, and thus likely made the locus more prone to off target activity by TAS4b gRNA. Thus, our findings underscore the importance of exploring for off-target effects in vivo especially when rootstocks and/or cultivars other than the reference genome are subject matter for genome editing experiments. It is worth noting that the off-target effect of *TAS4b* gRNA on the *TAS4a* locus was fortuitous and to our advantage wherein we successfully edited two homologous loci of interest with a single guide RNA.

Due to genetic redundancy for *MYBA5/6/7* and *TAS4a/b/c* loci, it remains to be determined whether the events characterized here will have visible phenotypes impacting anthocyanin pigmentation and/or PD resistance/tolerance. Future experiments can employ multiple guide constructs (Jacobs and Martin 2016) to target all *MYBA* and *TAS4* family members, and to target the sole *Vvi-MIR828* locus at locations upstream or downstream of the mature miR828 in the hairpin structure to generate leaky dominant-negative alleles predicted to alter DICER processing efficiency. Our initial test construct for *Vvi-MIR828* aimed to create null alleles by targeting the mature miR828 duplex per se but we failed to recover any regenerants, consistent with a speculated essential function of *MIR828*.

There is now scope with these novel edited materials to assess molecular phenotypes of deranged gene expression, pathogen resistance/tolerance, vector feeding preferences (Zeilinger et al. 2018), and GRBV systemic movement by agroinoculations (Yepes et al. 2018) on these genome-edited anthocyanin host effector grapevine lines. However, dissolution of the transgenic state or conversion to homozygosity of mutated haplotypes (like TAS4b-2-a2; Fig. 2c) and non-mutated wild type alleles (like TAS4b-1) to obtain desired homozygous plants by back-crossing and genetic segregation of sexually reproduced individual progeny is a lengthy route. This is because of the difficulty to obtain successful crosses of grapevine with greenhouse material, and several years of time elapsed before regenerated field-grown juvenile transgenic plants will flower. Further work on fundamental processes of plant interactions with GRBV and XF can leverage translational science from model organisms to crops, with potential for broad impacts on agriculture, development of sustainable nutrient management tools, and understanding the mechanisms of pathogen resistance/tolerance and pleiotropic disease states.

Conclusions

CRISPR-cas9 technology has been successfully used to knock-down several protein coding genes in several plant species. Although successful editing of non-coding RNAs has been demonstrated in animals [reviewed in Basak and Nithin (2015)], the only report on editing of a non-coding RNA is in tomato (Li et al. 2018). We demonstrate successful gene editing of a non-coding regulatory RNA *TAS4a/b* in grape cultivar 101-14, an anthocyanin producing rootstock. We further demonstrate fortuitous off-target effects of TAS4b guide RNA on *TAS4a* locus resulting in a chimeric TAS4a-b locus subject to homologous recombination events associated with off-target editing. Future studies are now possible to test the roles of *Vvi-MYBA7* and *Vvi-TAS4a/b/c* in tissue-specific anthocyanin expression and the genes' roles in microbe and virus disease etiologies and possibly feeding preferences of arthropod vectors (Zeilinger et al. 2018).

Acknowledgements The authors thank David Tricoli, UC Davis Plant Transformation Facility, for gift of Agrobacterium strain EHA105 and for grapevine transformations and regeneration, and Tom Jacobs, VIB-UGent Center for Plant Systems Biology, for advice on CRISPR vector constructions and assays.

Author contributions CDR and SS designed and performed the research, analyzed data, and wrote the manuscript.

Funding This project was supported by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, Agreement Numbers 15-0214-SA and 17-0514-SA. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Costs of Open Access publication were defrayed by a gift to the TTU Foundation from Richard Bagdasarian, Inc., by TTU Department of Biological Sciences, and TTU Vice President for Research Open Access Publication Initiative.

Availability of data and materials The sequencing runs were submitted as raw fastq files to NCBI Sequence Read Archive with Bioproject accession PRJNA602781. Authors will freely share transgenic events and other materials described in this article via a Materials Transfer Agreement promulgated by the Texas Tech University Office of Technology Commercialization (TTU-OTC). All data needed to evaluate the conclusions in the paper are present in the paper. Additional data related to this paper may be requested from the authors.

Compliance with ethical standards

Conflict of interest The authors declare they have no financial or competing conflict of interests.

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