JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comparison of Sugar, Acids, and Volatile Composition in Raspberry Bushy Dwarf Virus-Resistant Transgenic Raspberries and the Wild Type 'Meeker' (*Rubus Idaeus* L.)

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Raspberry bushy dwarf virus (RBDV) causes a significant reduction in yield and quality in raspberry and raspberry−blackberry hybrid. Genetic modifications were made to 'Meeker' red raspberries to impart RBDV resistance. The RBDV-resistant transgenic and wild type 'Meeker' plants were grown in Oregon and Washington, and the fruits were harvested in the 2004 and 2005 growing seasons. Year-to-year and site-to-site variations were observed for the °Brix and titratable acidity, with Oregon raspberries having slightly higher °Brix and lower titratable acidity than Washington raspberries. Twenty-nine volatile compounds were quantified using stir bar sorptive extraction (SBSE) paired with gas chromatography−mass spectrometry (GC-MS). There were very few differences in volatile concentrations between the transgenic varieties and the wild type 'Meeker'. Much larger variations were observed between sites and harvest seasons. Raspberries grown in Oregon appeared to have higher concentrations of δ-octalactone, δ-decalactone, geraniol, and linalool. Chiral analysis of α-ionone, α-pinene, linalool, terpinen-4-ol, δ-octalactone, and δ-decalactone demonstrated a much higher percentage of one isomer over the other, particularly α-ionone, α-pinene, δ-octalactone, and δ-decalactone, with more than 90% of one isomer, while a racemic mixture was observed for linalool. The isomeric analysis revealed very little variation between varieties, locations, or years. The flavor compounds tested in this study did not show any difference between the transgenic lines and the wild type 'Meeker' raspberry.

KEYWORDS: Raspberry; Meeker; RBDV-resistant; transgenic; volatile; SBSE

INTRODUCTION

Raspberries, commonly grouped with blackberries and blackberry−raspberry hybrids as "brambles" and "caneberries", grow naturally in all temperate regions of the northern hemisphere and are grown commercially in the United States, Europe, Chile, New Zealand, and Australia (*1*).

The Pacific Northwest, made up of Oregon and Washington states, produces the largest volume of red raspberries in the United States. This area has ideal growing conditions for red raspberries. The production of the raspberry has increased dramatically over the last fifteen years due to the implementation of machine harvestable and high yield cultivars (*2*). Since the early 1980s, 'Meeker' has begun to replace 'Willamette' and is now the leading red raspberry cultivar grown commercially in the Pacific Northwest, accounting for 80% of the red raspberry acreage in Washington state (*2*). The 'Meeker' variety is popular due to high yields, a long harvest season, resistance to root rot, and machine harvest characteristics (*2*). 'Meeker' fruit has a desirable color, firm texture, and good sensorial qualities, including aroma, sweetness, and acidity.

With the change in cultivars and increased planting density, there has been a rapid increase in the incidence of raspberry bushy dwarf virus (RBDV). RBDV, genus *Idaeovirus*, occurs naturally worldwide and can infect most bramble species and cultivars (*3–6*). RBDV is a pollen- and seed-borne virus that is commonly found in red and black raspberry. RBDV causes dwarfing and shoot proliferation (*5*), reduces cane vigor, and causes leaf discoloration, known as interveinal chlorosis, in combination with other diseases, such as the black raspberry necrosis. The primary symptom of raspberry bushy dwarf virus in both red and black raspberries is crumbly fruit. In red raspberries, it induces drupelet abortion and decreases drupelet set leading to crumbly fruit and reduced yield. Decreased drupelet set results in smaller berries that do not hold together. These berries can only be sold for processing as juice, puree, or jam, which reduces the value of the crop.

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10.1021/jf800253e CCC: $40.75
Published on Web 07/04/2008

Table 1. °Brix, Titratable Acidity, pH, and °Brix/TA Ratio for Wild Type and Transgenic Raspberries Grown in Oregon and Washington during 2004 and 2005

location	°Brix	titratable acidity	original pH	°Brix/ TA ratio
wild type 'Meeker', Lynden, WA, 2004	12.9	1.13	3.1	11.36
transgenic 2171 BJ, Lynden, WA, 2004	12.1	1.11	3.2	10.89
transgenic 2172 AG, Lynden, WA, 2004	13.6	1.17	3.1	11.61
transgenic 2174 BO, Lynden, WA, 2004	13.6	1.22	3.2	11.16
transgenic 2174 BS, Lynden, WA, 2004	13.0	1.16	3.2	11.19
wild type 'Meeker', Aurora, OR, 2004	13.7	1.24	3.0	11.01
transgenic 2171 BJ, Aurora, OR, 2004	14.9	0.96	3.0	15.45
transgenic 2172 AG, Aurora, OR, 2004	13.9	0.95	3.0	14.63
transgenic 2172 BJ, Aurora, OR, 2004	15.2	1.09	3.0	13.91
transgenic 2174 BO, Aurora, OR, 2004	14.6	1.11	3.0	13.16
transgenic 2174 BS, Aurora, OR, 2004	15.9	1.14	3.0	13.99
wild type 'Meeker', Lynden, WA, 2005	10.8	1.63	2.6	6.63
transgenic 2171 BJ, Lynden, WA, 2005	10.5	1.52	2.6	6.93
transgenic 2172 AG, Lynden, WA, 2005	10.4	1.50	2.6	6.88
transgenic 2172 BJ, Lynden, WA, 2005	10.4	1.43	2.7	7.26
transgenic 2174 BO, Lynden, WA, 2005	11.0	1.59	2.6	6.90
transgenic 2174 BS, Lynden, WA, 2005	10.7	1.55	2.6	6.85
wild type 'Meeker', Aurora, OR, 2005	13.4	1.24	2.8	10.78
transgenic 2171 BJ, Aurora, OR, 2005	13.7	1.32	2.9	10.32
transgenic 2172 AG, Aurora, OR, 2005	13.4	1.03	2.9	12.98
transgenic 2172 BJ, Aurora, OR, 2005	13.7	1.33	2.8	10.27
transgenic 2174 BO, Aurora, OR, 2005	13.2	1.28	2.8	10.29
transgenic 2174 BS, Aurora, OR, 2005	13.4	1.35	2.8	9.85

RBDV is transmitted through pollen and seeds from infected plants and can spread rapidly through fields (*2, 5*). Because of its pollen- and seed-borne nature, RBDV spreads during bloom. When a raspberry flower is pollinated with RBDV infected pollen, both the embryo and the mother plant can become infected. The exact mechanism by which RBDV infects the seed and mother plant is unknown. There are many possible factors that could affect transmission including temperature and humidity levels, insect vectors, and the spread from native vegetation. Studies have already shown that native *Rubus* species such as thimbleberry (*R. parviflorus*) can be naturally infected with RBDV. This species is commonly found growing around raspberry fields in the Pacific Northwest and could provide a source of RBDV inoculum. Following the initial infection, a field can be completely infected within five years. An infected raspberry field requires that the plants, trellising, and irrigation system are removed followed by fumigation and replanting with new plants. Resistant cultivars are currently the only effective method of RBDV control in areas where there is inoculum present (*7, 8*).

The best way to avoid infection from the RBDV type strain is to plant resistant cultivars. There are several red raspberry cultivars available that are resistant to RBDV from both North America and Europe. Unfortunately, these are often unable to match the yield and quality of many RBDV susceptible cultivars.

Conventional breeding methods have difficulties when attempting to produce resistant, commercially viable plants (*5*). These difficulties in producing resistance through conventional breeding methods have led to newer research to engineer pathogen derived resistance to RBDV in 'Meeker' red raspberries. Pathogen derived resistance makes use of viral genes or mutated viral genes inserted into the host plant, which subsequently interfere with the virus life cycle.

Flavors in rubes and ribes are mainly formed during a brief ripening period and are influenced by numerous factors. These factors include internal genetic makeup and external agronomical factors, such as climate and soil type, as well as the ripeness and handling of the fruit (*1, 9–12*). Sugars and organic acids are strongly affected by climate variations. Volatile aroma compounds are affected by various environmental factors due to effects on precursors and enzymatic activity within the fruit (*12, 13*).

Volatile compounds are the secondary metabolites found in plants, and they are generated through numerous pathways during the fruit ripening process. Volatile variations due to genetic makeup of cultivars are common, although the significance of these variations depends on the cultivars and flavor compounds (*10, 14, 15*). The objective of this study is to determine if the sugar, acid, and volatile composition of RBDV-resistant lines will vary from the wild-type 'Meeker' raspberry.

MATERIALS AND METHODS

Chemicals. Hexanal, (*E*)-2-hexenal, nerol, α-phellandrene, α-ionone, β-ionone, *p*-cymene, ethyl hexanoate, δ-decalactone, γ-nonalactone, and linalool were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). 2-Heptanone, 2-nonanone, 2-nonanol, α-pinene, γ- terpinene, geraniol, α-terpinene, limonene, α-terpineol, and myrcene were obtained from K&K Laboratories (Jamaica, NY). (*Z*)-3-Hexenol and (*Z*)-3-hexenyl acetate were obtained from Bedoukian Research (Danbury, CT). Terpinen-4-ol was obtained from TCI Japan (Tokyo, Japan). Methyl nonanoate was obtained from Eastman Blue (Rochester, NY). δ-Octalactone was obtained from Alfa Aesar (Ward Hill, MA). *cis*-Jasmone was purchased from Pfalz & Bauer (Waterbury, CT).

Developing RBDV-Resistant Plants. The RBDV resistance was developed through directed mutagenesis of the movement protein in the virus and transformation of the mutated protein to 'Meeker' raspberries, preventing the cell to cell movement of the virus within the raspberry plant (*2*). Five RBDV movement protein constructs were developed and used for transformation including: (1) nontranslatable RNA that lacked the AUG start codon and had two stop codons introduced 3 and 6 codons downstream, (2) the amino acids 2−6 deleted, (3) the amino acids 5−14 deleted, (4) the amino acids 37−42 deleted, and (5) wild-type movement protein. In addition, the wild-type CP gene was also used. The mutations were confirmed by sequence analysis and cloned into the binary vector pAG2170 between the 35S promoter from cassava vein mosaic virus (CsVMV) and the NOS terminator. The plasmids also contained the *hpt* selectable marker gene for hygomycin resistance. This plasmid was then used to transform *Agrobacterium tumefaciens*, which was used to transform the 'Meeker' red raspberry. Transformation was carried out using leaf pieces and petioles from tissue cultured raspberry plantlets. Approximately 50 individual lines were regenerated with each construct.

Table 2. Volatile Concentrations (μg/kg) for Wild Type and Transgenic 'Meeker' Red Raspberries Grown in Lynden, Washington, during 2004

compd	wild type	transgenic 2171 BJ	transgenic 2172 AG	transgenic 2172 BJ	transgenic 2174 BO	transgenic 2174 BS
(*Z*)-3-hexenol	145 ± 8	137 ± 2	188 ± 9	177 ± 6	161 ± 5	158 ± 7
cumic alcohol	23 ± 1	17 ± 1	24 ± 4	29 ± 2	48 ± 1	24 ± 0.2
6-methyl-5-hepten-2-ol	79 ± 5	85 ± 3	71 ± 9	78 ± 3	102 ± 7	84 ± 3
2-nonanol	3 ± 0.1	2 ± 0.0	2 ± 1	2 ± 0.1	13 ± 0.2	3 ± 0.1
hexanal	182 ± 14	234 ± 14	123 ± 31	155 ± 10	408 ± 118	178 ± 10
(*E*)-2-hexenal	420 ± 18	420 ± 48	221 ± 114	394 ± 15	600 ± 16	374 ± 17
(*Z*)-3-hexenyl acetate	5 ± 0.2	4 ± 0.0	10 ± 1	10 ± 0.4	4 ± 0.2	6 ± 0.3
ethyl hexanoate	5 ± 1	3 ± 0.1	2 ± 1	3 ± 0.2	3 ± 1	7 ± 0.3
methyl nonanoate	1 ± 0.1	1 ± 0.1	1 ± 0.4	2 ± 0.2	1 ± 0.4	1 ± 0.0
2-heptanone	85 ± 2	79 ± 3	51 ± 20	83 ± 4	67 ± 17	123 ± 10
2-nonanone	26 ± 1	24 ± 0.2	17 ± 2	28 ± 1	17 ± 1	28 ± 1
zingerone	59 ± 17	48 ± 9	146 ± 10	121 ± 34	51 ± 3	77 ± 8
δ-octalactone	484 ± 19	415 ± 15	270 ± 41	312 ± 10	303 ± 24	526 ± 13
δ-decalactone	540 ± 14	469 ± 6	401 ± 26	441 ± 1	326 ± 8	552 ± 7
p-cymene	14 ± 1	10 ± 1	6 ± 4	14 ± 1	4 ± 1	12 ± 1
geraniol	121 ± 4	123 ± 3	113 ± 4	149 ± 6	95 ± 8	129 ± 3
α-ionone	80 ± 2	63 ± 1	63 ± 5	84 ± 4	62 ± 3	71 ± 1
β-ionone	93 ± 1	75 ± 1	72 ± 2	83 ± 5	71 ± 1	87 ± 1
limonene	2 ± 0.1	1 ± 1	1 ± 1	2 ± 0.1	1 ± 0.4	1 ± 0.3
linalool	8 ± 0.4	6 ± 0.1	8 ± 1	8 ± 0.2	7 ± 0.3	7 ± 0.1
myrcene	5 ± 5	10 ± 4	6 ± 1	7 ± 3	3 ± 6	7 ± 6
nerol	15 ± 1	12 ± 0.2	14 ± 1	17 ± 1	6 ± 0.4	17 ± 0.3
α-phellandrene	28 ± 25	34 ± 4	30 ± 4	30 ± 26	11 ± 3	53 ± 3
α-pinene	29 ± 0.2	20 ± 1	25 ± 5	29 ± 2	10 ± 2	25 ± 1
sabinene	16 ± 1	12 ± 1	12 ± 6	17 ± 1	6 ± 0.4	17 ± 1
α-terpinene	4 ± 3	8 ± 1	8 ± 4	17 ± 1	4 ± 1	12 ± 1
γ-terpinene	11 ± 1	10 ± 0.1	8 ± 5	21 ± 1	5 ± 0.4	14 ± 0.2
α-terpineol	12 ± 1	9 ± 0.1	13 ± 1	11 ± 0.4	6 ± 0.3	11 ± 0.3
terpinen-4-ol	96 ± 4	81 ± 1	113 ± 5	124 ± 4	47 ± 2	104 ± 2

Table 3. Volatile Concentrations (μg/kg) for Wild Type and Transgenic 'Meeker' Red Raspberries Grown in Aurora, Oregon, during 2004

compd	wild type	transgenic 2171 BJ	transgenic 2172 AG	transgenic 2172 BJ	transgenic 2174 BO	transgenic 2174 BS
(*Z*)-3-hexenol	190 ± 2	136 ± 4	152 ± 3	203 ± 9	117 ± 5	131 ± 5
cumic alcohol	30 ± 0.2	24 ± 1	34 ± 1	11 ± 1	39 ± 0.2	25 ± 1
6-methyl-5-hepten-2-ol	92 ± 0.2	82 ± 4	83 ± 2	87 ± 3	66 ± 4	77 ± 4
2-nonanol	4 ± 0.2	6 ± 0.2	5 ± 0.1	2 ± 0.1	3 ± 0.3	9 ± 0.1
hexanal	90 ± 3	155 ± 21	105 ± 5	137 ± 10	89 ± 4	146 ± 10
(*E*)-2-hexenal	289 ± 7	358 ± 16	240 ± 1	473 ± 29	272 ± 14	277 ± 7
(*Z*)-3-hexenyl acetate	3 ± 0.2	2 ± 0.1	4 ± 0.1	6 ± 0.1	2 ± 0.2	3 ± 0.1
ethyl hexanoate	5 ± 0.2	14 ± 1	10 ± 0.4	6 ± 1	5 ± 0.4	25 ± 0.4
2-heptanone	63 ± 2	64 ± 3	53 ± 1	74 ± 5	40 ± 3	94 ± 3
2-nonanone	22 ± 1	11 ± 1	18 ± 0.3	15 ± 1	10 ± 0.4	20 ± 1
zingerone	109 ± 7	219 ± 15	92 ± 7	127 ± 12	173 ± 11	291 ± 62
δ-octalactone	546 ± 11	785 ± 36	576 ± 22	361 ± 8	781 ± 5	1049 ± 14
δ-decalactone	602 ± 3	706 ± 6	561 ± 4	396 ± 11	668 ± 10	841 ± 20
p-cymene	12 ± 0.2	13 ± 1	14 ± 1	8 ± 1	9 ± 1	18 ± 0.3
geraniol	167 ± 7	149 ± 3	133 ± 0	188 ± 2	127 ± 7	161 ± 8
α-ionone	63 ± 1	57 ± 4	77 ± 2	60 ± 4	62 ± 2	75 ± 1
β-ionone	76 ± 2	75 ± 4	74 ± 2	63 ± 3	79 ± 1	88 ± 2
limonene	2 ± 0.1	1 ± 0.1	1 ± 0.0	1 ± 0.1	1 ± 0.1	1 ± 0.1
linalool	12 ± 0.4	18 ± 0.4	13 ± 0.0	11 ± 0.3	12 ± 1	16 ± 1
myrcene	6 ± 0.3	6 ± 3	3 ± 0.6	5 ± 4	3 ± 0.1	6 ± 2
nerol	20 ± 1	17 ± 0.3	14 ± 0.0	13 ± 0.1	13 ± 1	19 ± 1
α-phellandrene	42 ± 7	30 ± 2	23 ± 3	27 ± 1	21 ± 2	43 ± 3
α-pinene	25 ± 1	16 ± 2	15 ± 1	17 ± 1	13 ± 2	20 ± 1
sabinene	15 ± 1	9 ± 0	8 ± 0.4	9 ± 1	7 ± 0.3	16 ± 0.2
α-terpinene	9 ± 1	9 ± 1	7 ± 0.1	4 ± 0	3 ± 0.1	10 ± 4
γ-terpinene	8 ± 0.4	7 ± 1	6 ± 0.2	3 ± 0.1	1 ± 0.0	13 ± 1
α-terpineol	16 ± 0.2	18 ± 0.3	16 ± 0.2	12 ± 0.1	16 ± 1	18 ± 1
terpinen-4-ol	104 ± 3	82 ± 2	66 ± 0.2	70 ± 1	51 ± 2	105 ± 6

Each individual line was then propagated for greenhouse and field trials. Each line was graft inoculated with RBDV; lines that remained free of RBDV after grafting were regrafted two additional times. Four 3-plant plots were established for each transgenic line in areas of extreme disease pressure. Each plot was tested for the presence of RBDV annually, until it tested positive or up until six years. Plant type and fruit quality were assessed based on visual characteristics until plants became infected or for six years.

Table 4. Volatile Concentrations (μg/kg) for Wild Type and Transgenic 'Meeker' Red Raspberries Grown in Lynden, Washington, during 2005

compd	wild type	transgenic 2171 BJ	transgenic 2172 AG	transgenic 2172 BJ	transgenic 2174 BO	transgenic 2174 BS
(*Z*)-3-hexenol	149 ± 8	141 ± 4	168 ± 7	152 ± 24	153 ± 10	188 ± 18
cumic alcohol	45 ± 1	43 ± 2	32 ± 2	57 ± 1	37 ± 16	34 ± 3
6-methyl-5-hepten-2-ol	63 ± 5	54 ± 2	66 ± 1	56 ± 3	57 ± 3	69 ± 7
2-nonanol	3 ± 0.1	5 ± 0.1	3 ± 0.3	10 ± 0.3	3 ± 0.0	4 ± 0.1
hexanal	132 ± 3	120 ± 5	126 ± 16	133 ± 15	101 ± 8	213 ± 14
(*E*)-2-hexenal	416 ± 22	349 ± 4	285 ± 15	365 ± 15	309 ± 14	538 ± 45
(*Z*)-3-hexenyl acetate	6 ± 1	5 ± 0.2	7 ± 0.0	5 ± 0.4	5 ± 0.1	5 ± 0.3
ethyl hexanoate	6 ± 0.4	8 ± 0.3	7 ± 1	8 ± 0.3	8 ± 0.4	6 ± 0.2
2-heptanone	84 ± 6	96 ± 5	66 ± 46	102 ± 2	88 ± 8	117 ± 7
2-nonanone	20 ± 2	44 ± 2	22 ± 0.1	49 ± 3	24 ± 0.9	26 ± 1
zingerone	220 ± 13	187 ± 48	291 ± 99	350 ± 29	177 ± 27	236 ± 102
δ-octalactone	375 ± 14	438 ± 22	327 ± 20	493 ± 12	418 ± 4	395 ± 41
δ-decalactone	476 ± 24	498 ± 6	433 ± 8	523 ± 9	497 ± 6	462 ± 30
p-cymene	20 ± 2	20 ± 2	10 ± 2	24 ± 2	18 ± 2	16 ± 1
geraniol	126 ± 12	121 ± 8	131 ± 1	148 ± 7	123 ± 7	132 ± 9
α-ionone	89 ± 59	82 ± 2	53 ± 1	75 ± 3	75 ± 0.1	80 ± 2
β-ionone	94 ± 5	94 ± 3	72 ± 1	96 ± 4	88 ± 0.4	90 ± 2
limonene	2 ± 0.2	2 ± 0.1	1 ± 0.1	2 ± 0.2	2 ± 0.2	2 ± 0.2
linalool	14 ± 1	10 ± 0.2	10 ± 0.2	12 ± 0.3	13 ± 0.4	12 ± 1
myrcene	5 ± 1	4 ± 1	16 ± 8	4 ± 1	9 ± 7	2 ± 2
nerol	21 ± 3	20 ± 2	21 ± 1	29 ± 2	20 ± 1	20 ± 2
α-phellandrene	57 ± 16	51 ± 10	33 ± 19	72 ± 46	59 ± 25	54 ± 25
α-pinene	25 ± 2	28 ± 1	30 ± 3	39 ± 2	34 ± 3	29 ± 1
sabinene	22 ± 3	24 ± 2	15 ± 7	37 ± 1	23 ± 1	23 ± 2
α-terpinene	23 ± 2	22 ± 0.4	10 ± 5	40 ± 10	20 ± 1	21 ± 2
γ-terpinene	24 ± 2	21 ± 1	6 ± 7	49 ± 4	20 ± 2	24 ± 1
α-terpineol	18 ± 1	16 ± 0.4	14 ± 0.1	22 ± 1	19 ± 1	17 ± 2
terpinen-4-ol	135 ± 14	145 ± 7	139 ± 2	214 ± 9	138 ± 7	158 ± 12

Table 5. Volatile Concentrations (μg/kg) for Wild Type and Transgenic 'Meeker' Red Raspberries Grown in Aurora, Oregon, during 2005

compd	wild type	transgenic 2171 BJ	transgenic 2172 AG	transgenic 2172 BJ	transgenic 2174 BO	transgenic 2174 BS
(*Z*)-3-hexenol	249 ± 18	204 ± 13	218 ± 3	189 ± 8	210 ± 25	192 ± 2
cumic alcohol	64 ± 2	55 ± 10	50 ± 2	64 ± 4	63 ± 4	61 ± 2
6-methyl-5-hepten-2-ol	107 ± 6	81 ± 12	103 ± 7	84 ± 6	86 ± 10	96 ± 1
2-nonanol	7 ± 0.4	5 ± 1	6 ± 0.3	6 ± 0.1	8 ± 0.2	7 ± 0.1
hexanal	170 ± 17	175 ± 32	176 ± 6	126 ± 8	168 ± 16	155 ± 5
(*E*)-2-hexenal	425 ± 31	385 ± 210	468 ± 0.7	467 ± 11	481 ± 26	477 ± 11
(*Z*)-3-hexenyl acetate	11 ± 1	4 ± 1	5 ± 0.4	6 ± 0.2	6 ± 15	7 ± 0.3
ethyl hexanoate	11 ± 0.7	6 ± 2	2 ± 0.1	8 ± 0.1	12 ± 2	10 ± 1
methyl nonanoate	1 ± 0.2	1 ± 1	1 ± 0.4	1 ± 0.2	1 ± 0.4	1 ± 0.1
2-heptanone	108 ± 8	59 ± 39	87 ± 1	87 ± 4	85 ± 7	105 ± 6
2-nonanone	36 ± 2	24 ± 3	34 ± 1	31 ± 2	33 ± 2	33 ± 2
zingerone	234 ± 44	181 ± 22	160 ± 10	218 ± 20	182 ± 37	206 ± 35
δ-octalactone	547 ± 24	542 ± 63	446 ± 13	583 ± 14	518 ± 53	595 ± 5
δ-decalactone	625 ± 22	740 ± 275	516 ± 5	627 ± 10	591 ± 24	622 ± 6
p-cymene	24 ± 3	13 ± 11	7 ± 7	23 ± 1	22 ± 1	23 ± 2
geraniol	155 ± 6	163 ± 13	161 ± 1	166 ± 8	166 ± 11	150 ± 7
α-ionone	53 ± 3	63 ± 23	56 ± 1	54 ± 2	52 ± 2	59 ± 1
β-ionone	73 ± 3	84 ± 35	66 ± 1	70 ± 1	72 ± 3	74 ± 1
limonene	2 ± 0.4	1 ± 1	2 ± 0.2	2 ± 0.1	2 ± 0.2	2 ± 0.4
linalool	15 ± 1	16 ± 1	16 ± 1	17 ± 1	20 ± 2	17 ± 1
myrcene	4 ± 5	9 ± 2	23 ± 1	13 ± 6	15 ± 12	20 ± 6
nerol	27 ± 2	21 ± 4	25 ± 0.2	25 ± 1	27 ± 1	25 ± 1
α-phellandrene	49 ± 20	44 ± 12	61 ± 6	58 ± 46	45 ± 17	59 ± 8
α-pinene	33 ± 3	31 ± 5	26 ± 0.5	28 ± 1	28 ± 3	27 ± 1
sabinene	30 ± 4	14 ± 0	21 ± 3	25 ± 1	23 ± 2	23 ± 3
α-terpinene	25 ± 5	13 ± 4	15 ± 2	20 ± 2	17 ± 2	19 ± 2
γ-terpinene	25 ± 3	13 ± 9	14 ± 1	23 ± 3	6 ± 6	18 ± 2
α-terpineol	22 ± 1	20 ± 1	19 ± 1	23 ± 1	25 ± 2	21 ± 1
terpinen-4-ol	172 ± 7	128 ± 37	137 ± 2	162 ± 9	161 ± 11	153 ± 7

Red Raspberry Samples. Ripe wild type and infection-free transgenic 'Meeker' raspberries were harvested from Lynden, Washington, in July 2004 and 2005 and Aurora, OR, in June 2004 and 2005, when the fruits were fully ripe. The fruits from both the transgenic lines and the wild type were harvested at the same time for accurate comparison. The fruits were transported, chilled, to the laboratory, where they were quickly individually quick frozen (IQF) and stored at −37 °F until analyses were performed.

°Brix and Titratable Acidity. Red raspberries (100 g) were thawed at room temperature for 3 h. The juice from the thawed berry was

Table 6. Chiral Percentages for Wild Type and Transgenic 'Meeker' Raspberries Grown in Washington and Oregon during 2004

compd	(*R*)-α-ionone	(*S*)-α-ionone	(*R*)-α-pinene	(*S*)-α-pinene	(*R*)-linalool	(*S*)-linalool	(*R*)-terpinen-4-ol	(*S*)-terpinen-4-ol	(*S*)-δ-octalactone	(*R*)-δ-octalactone	(*S*)-δ-decalactone	(*R*)-δ-decalactone
wild type 'Meeker', Lynden, WA, 2004	100	0	100	0	46.9	53.1	20.1	79.9	95.6	4.4	99.3	0.7
transgenic 2171 BJ, Lynden, WA, 2004	98.9	1.1	100	0	46.9	53.1	20.7	79.3	100	0	99.0	1.0
transgenic 2172 AG, Lynden, WA, 2004	98.2	1.8	100	0	45.8	54.2	20.4	79.6	100	0	99.2	0.8
transgenic 2172 BJ, Lynden, WA, 2004	98.9	1.1	100	0.0	46.1	53.9	20.9	79.1	100	0	99.1	0.9
transgenic 2174 BO, Lynden, WA, 2004	98.9	1.1	100	0.0	45.3	54.7	19.9	80.1	100	0	99.3	0.7
transgenic 2174 BS, Lynden, WA, 2004	98.7	1.3	100	0.0	45.2	54.8	20.1	79.9	100	0	99.3	0.7
wild type 'Meeker', Aurora, OR, 2004	99	1.0	100	0.0	44.7	55.3	20.8	79.2	96.1	3.9	99.2	0.8
transgenic 2171 BJ, Aurora, OR, 2004	100	0.0	100	0.0	44.4	55.6	20.8	79.2	100	0	99.2	0.8
transgenic 2172 AG, Aurora, OR, 2004	100	0.0	100	0.0	47.7	52.3	20.8	79.2	100	0	99.2	0.8
transgenic 2172 BJ, Aurora, OR, 2004	100	0.0	100	0.0	49.3	50.7	21.1	78.9	100	0	99.3	0.7
transgenic 2174 BO, Aurora, OR, 2004	98.2	1.8	100	0.0	47.8	52.2	20.5	79.5	100	0	99.3	0.7
transgenic 2174 BS, Aurora, OR, 2004	99.0	1.0	100	0.0	47.5	52.5	20.9	79.1	100	0	99.1	0.9

Table 7. Chiral Percentages for Wild Type and Transgenic 'Meeker' Raspberries Grown in Washington and Oregon during 2005

compd	(*R*)-α-ionone	(*S*)-α-ionone	(*R*)-α-pinene	(*S*)-α-pinene	(*R*)-linalool	(*S*)-linalool	(*R*)-terpinen-4-ol	(*S*)-terpinen-4-ol	(*S*)-δ-octalactone	(*R*)-δ-octalactone	(*S*)-δ-decalactone	(*R*)-δ-decalactone
wild type 'Meeker', Lynden, WA, 2005	98.8	1.2	100	0	47.8	52.2	20.3	79.7	96.4	3.6	99.1	0.9
transgenic 2171 BJ, Lynden, WA, 2005	98.1	1.9	100	0	48.3	51.7	21.0	79.0	100	0	99.0	1.0
transgenic 2172 AG, Lynden, WA, 2005	97.4	2.6	100	0	42.4	57.6	21.0	79.0	100	0	99.1	0.9
transgenic 2172 BJ, Lynden, WA, 2005	98.2	1.8	100	0	42.1	57.9	20.6	79.4	100	0	99.2	0.8
transgenic 2174 BO, Lynden, WA, 2005	98.2	1.8	100	0	45.5	54.5	20.1	79.9	100	0	99.3	0.7
transgenic 2174 BS, Lynden, WA, 2005	97.9	2.1	100	0	44.0	56.0	20.3	79.7	100	0	99.2	0.8
wild type 'Meeker', Aurora, OR, 2005	98.7	1.3	100	0	45.0	55.0	21.3	78.7	96.1	3.9	99.2	0.8
transgenic 2172 AG, Aurora, OR, 2005	98.5	1.5	100	0	41.5	58.5	21.5	78.5	100	0	99.2	0.8
transgenic 2172 BJ, Aurora, OR, 2005	98.3	1.7	100	0	40.3	59.7	21.3	78.7	100	0	99.0	1.0
transgenic 2174 BO, Aurora, OR, 2005	98.4	1.6	100	0	41.6	58.4	21.2	78.8	100	0	99.2	0.8
transgenic 2174 BS, Aurora, OR, 2005	98.6	1.4	100	0	43.4	56.6	21.0	79.0	100	0	99.4	0.6

analyzed for °Brix using a Pocket Pal-1 pocket refractometer from ATAGO (Bellevue, WA). The berries were then blended with 50 mL of boiling distilled water at high speed in a blender for 30 s. This mixture was then placed in a boiling water bath for 5 min to deactivate enzymatic activity. The berry mixture was then centrifuged at 2000 rpm for 20 min, and the supernatant was collected for further analysis. A portion of the juice (7 mL) collected above was combined with 50 mL of CO_2 free water and then titrated with 0.1N NaOH solution to an end point of pH 8.1. The results were reported as percent (%) of citric acid.

Extraction of Volatile Compounds Using Stir Bar Sorptive Extraction. Red raspberries (150 g) were thawed for 3 h. The berries were blended with 1% $CaCl_2$ and 10% NaCl in a commercial blender for 30 s. The calcium chloride was added to inhibit enzyme activity, and the sodium chloride was added to increase recovery of extraction (*16*). The mixture was centrifuged at 2000 rpm for 20 min, and the supernatant was collected.

A stir bar sorptive extraction (SBSE) stir bar (1 cm long, 0.5 mm film thickness, Gerstel USA, Baltimore, MD) with a polydimethylsiloxane (PDMS) phase was used for the extraction of volatile compounds. The stir bar was cleaned with 80% acetonitrile in methanol overnight, allowed to air-dry for 1 h, and then conditioned for 45 min at 300 °C with a 50 mL/min nitrogen flow. Juice samples (10 g) were weighed into a 20 mL clear glass vial (I-Chem, New Castle, DE), and 10 μL of internal standard mixture in methanol was added. The juice was extracted at room temperature for 1 h at 1000 rpm. All samples were analyzed in triplicate.

Gas Chromatography−Mass Spectrometry. The analysis of volatile compounds was carried out by using an Agilent 6890 gas chromatograph equipped with a 5973 mass selective detector (Agilent

Techonologies, Inc., Wilmington, DE) and a Gerstel MPS-2 multipurpose TDU autosampler with a CIS-4 cooled injection system (Gerstel USA). The analytes were thermally desorbed at the TDU in splitless mode, ramping from 35 to 300 °C at a rate of 200 °C/min and held at the final temperature for 3 min. The CIS-4 was cooled to −80 °C with liquid nitrogen during the sample injection, then heated at 10 °C/second to 250 °C for 3 min. Solvent vent mode was used during the injection with a split vent flow of 50 mL/min beginning at 3 min. The helium column flow was 2.0 mL/min. Separation was achieved using a ZB-FFAP column (30 m × 0.32 mm I.D., 0.5 μm film thickness, Phenomenex, Torrance, CA). The GC temperature program was set at 40 °C for 2 min, ramped to 180 °C at a rate of 6 °C/min, then ramped to 240 °C at a rate of 4 °C/min and held for 20 min. Standard EI mode was used at 70 eV. The total mass ion chromatogram was obtained from 35 to 350 amu. System software control and data management/analysis were performed through Enhanced ChemStation Software (Agilent Techonologies, Inc.).

Chiral Ratio Analysis of Selected Volatile Compounds. The samples were extracted using the same procedure as described previously; however, internal standards were not added. Separation was achieved using a CyclosilB column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Agilent). The GC temperature program was set at 40 °C for 2 min, ramped to 90 °C at a rate 6 °C/min, then ramped to 135 °C at a rate of 1 °C/min, ramped to 220 °C at a rate of 10 °C/min and held for 5 min. Isomeric ratios were determined using the relative total mass ion abundance for each compound peak.

Quantification of Volatile Compound in Raspberry. The volatiles in raspberry fruits were quantified using stir bar sorptive extraction GC-MS method described previously (*17*). An internal standard solution was prepared in methanol containing 784 and 395 μg/mL of γ-nonalactone and *cis*-jasmone, respectively. An aliquot (5 μL) of this internal standard mixture was then added to 10 mL of water to yield a final concentration of 392 and 197 ppb, respectively. A stock solution of 2000 ppm of each compound (except zingerone) was prepared in methanol. The stock solution was then used to create calibration curve solutions in water with concentrations of 1000, 750, 500, 100, 50, 5, and 1 ng/mL with 5 μL of internal standard solution. The zingerone stock solutions was prepared at 45 000 ppm. The stock solution was used, with 5 μL of internal standard solution, to create calibration curve solutions in water with concentrations of 23, 17, 11, 2, 1, 0.1, and 0.02 μg/mL for zingerone. Mass spectrometry quantification was carried out using selective mass ions to avoid interference between coeluted compounds (*17*). Quantification curves were built by plotting selective ion abundance ratio of target compounds with their respective internal standards against the concentration ratio. The concentrations of volatile compounds in the samples were calculated based on the individual calibration curves.

RESULTS AND DISCUSSION

Five of the transgenic lines of 'Meeker' remained free of RBDV for the first three fruiting seasons in the field, while in the same field experiments there were 202 wild-type 'Meeker' plants that were all infected after three fruiting seasons. These five lines also remained free of RBDV after grafting three times in the greenhouse studies and were used for the detailed fruit analyses. At the end of six years of field trials, there was only one line that was still completely free of RBDV. The other four lines in this study had one or more of the plants in the field that had become infected with RBDV.

Wild type and transgenic 'Meeker' raspberries grown in Lynden, Washington, and Aurora, Oregon, were studied for two years. The average high temperatures in Lynden, Washington, in June and July (20 and 23 °C, respectively) are slightly cooler than those in Aurora, Oregon, (23 and 27 °C, respectively), while the temperatures at night are similar (9 and 11 °C) in Washington and (9 and 12 °C) in Oregon. The average rainfall for June and July in Washington (66 and 50.8 mm, respectively) is considerably higher than in Oregon (44.4 and 18.5 mm, respectively). These differences allow for the comparison between transgenic lines and wild type under different agronomic conditions.

°Brix and Titratable Acidity. The °Brix, titratable acidity, pH, and °Brix/TA ratio for the wild type and transgenic 'Meeker' raspberries grown in Washington and Oregon during 2004 and 2005 were presented in **Table 1**. Both year-to-year variations and site-to-site variations were observed. For both Washington and Oregon sites, fruits from 2004 had higher °Brix and lower acidity and pH than fruits from 2005 although the degree of difference was dependent on the sites. For year 2004, most of the raspberries grown in Washington had slightly lower °Brix and higher titratable acidity than the raspberries grown in Oregon. This trend was also observed in year 2005 and with a much greater magnitude. On average, raspberries grown in Oregon in 2005 had 30% higher °Brix and 20% lower titratable acidity than those grown in Washington. This difference correlated well with the climate difference whereby Oregon had a higher average temperature than Washington. Climate variations are known to affect the flavor of fruits during the growing and ripening seasons; warmer and drier weather generally produce fruit with higher sugar and lower acid contents (*1*).

Sugar and acid contents are also affected by fruit maturity. Sugar concentration typically increases drastically during fruit ripening and continues through the overripe stage (*18*). Acid concentration typically increases early in fruit development but decreases as the fruit ripens (*1*). Higher °Brix number is generally corresponded with a low titratable acidity and pH value (**Table 1**). Sugar, acid, and °Brix/TA ratio are often used as maturity indicators (*18*).

During both the 2004 and 2005 growing seasons, the °Brix for every transgenic variety and the wild type were very similar within a site and during one growing season. The °Brix values for all varieties grown in Washington in 2005 were within the range reported previously (*17*). The titratable acidity values for all transgenic varieties were similar to the wild type for that location and growing season.

Volatile Compound Comparison. Volatile compounds in raspberries were compared for all transgenic lines and the wild type in both Oregon and Washington, and the fruits for 2004 and 2005 were investigated. A total of 30 compounds were selected based on their previously reported importance to raspberry aroma (*9, 19–21*) as well as their representation to various chemical classes including alcohol, aldehyde, ketone, ester, and terpene and tepene alcohol. The concentration was estimated based on a standard curve and an internal standard. The volatile composition of the transgenic raspberries and the 'Meeker' was listed in **Tables 2−5**.

There were very few differences in volatile concentrations between the transgenic varieties and the wild type 'Meeker'. Most of the transgenic lines and the wild type 'Meeker' grown in Lynden, Washington, during 2004 had very similar volatile composition (**Table 2**). However, transgenic 2174 BO appeared to have higher hexanal and (*E*)-2-hexenal than the wild type 'Meeker' and the rest of the transgenic lines. Hexanal and (*E*)-2-hexenal are generated from enzymatic oxidative degradation of fatty acids (*13, 22*) by lipoxygenase (LOX) and are responsible for the "green" odor notes, and their concentrations are typically related with fruit maturity (*13, 22*).

Transgenic 2174 BO also had lower concentrations of most terpenoids than the wild type and the other transgenic lines (**Table 2**). Terpenoids are formed primarily through the isoprenoid pathway, starting from the condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by geranyl diphosphate synthase to form geranyl diphosphate (*13*). Further hydrolysis, cyclation, and/or oxidoreductions of geranyl diphosphate leads to the formation of various of terpenoids (*13*). However, this variation was not repeated for this variety grown in Oregon during 2004 (**Table 3**) or in Washington or Oregon during 2005 (**Tables 4** and **5**), indicating that this variation is likely not due to genetic variance. The volatile composition for all the transgenic lines and the wild type was very similar in Oregon during 2004 (**Table 3**).

In 2005, some of the volatile compounds had a wider range of variations in Washington (**Table 4**). Transgenic 2172 AG appeared to have lower (*E*)-2-hexenal, while transgenic 2174 BS had higher hexanal and (*E*)-2-hexenal; however, these variations were not seen in the Oregon grown varieties for the same year (**Table 5**), much less the 2004 raspberries (**Tables 2** and **3**). None of the variations were consistent to site or year. The results suggested that none of the transgenic line aroma volatiles were different from the wild type.

Much larger variations were observed between sites and harvest seasons. The raspberries grown in Washington in 2004 had similar hexanal and (*E*)-2-hexenal concentrations compared to the berries grown in Oregon, while they had much lower hexanal and (*E*)-2-hexenal in 2005. Volatile aroma compounds can increase, maintain steady levels, or decrease during the ripening process due to different pathways involved in volatile formation (*15*). Hexanal and (*E*)-2 hexenal can be generated from β-oxidation or lipoxygenase-catalyzed oxidative degradation of fatty acids (*13, 22–24*), and their concentrations in fruit typically decrease with maturity.

Raspberries grown in Oregon during 2005 also had lower α- and β-ionone than in Washington (**Tables 4** and **5**), which coincided with the higher concentrations of hexanal and (*E*)-2-hexenal. α- and β-Ionones are generated from the degradation of carotenoids, and their concentrations typically increase with maturity. The higher aldehydes and lower ionone concentrations suggest that the raspberries grown in Oregon in 2005 were less mature than the raspberries grown in Washington.

Raspberries grown in Oregon appeared to have higher concentrations of δ-octalactone, δ-decalactone, geraniol, and linalool. This trend was observed in both 2004 and 2005, indicating that these compounds are likely to be linked to agronomical differences between the locations, particularly the quantity and intensity of sunlight during the growing season (*1, 12*).

The concentrations of many aroma compounds such as esters and terpenoids generally increase in fruits with maturity and depend on the growing conditions, but the concentration at each stage and rate of change can vary between cultivars (*15*). Varying concentrations and activity of enzymes between cultivars is partially responsible for volatile variation; variations can also be due to growing conditions.

Chiral Analysis. Different isomers can have significantly different sensory thresholds and descriptors. Isomeric descriptors, distributions, thresholds, and variations have been studied in various natural products; however, possible variations due to growing conditions and locations have not been studied (*25–29*). Chemically produced compounds will generally have an equal, or racemic, mixture of all possible isomers; however because of stereospecific enzymes, compounds produced in plants will often favor one isomer over others (*29*).

The percent ratios for the compounds found during the chiral analysis, α-ionone, α-pinene, linalool, terpinen-4-ol, δ-octalactone, and δ-decalactone, were displayed in **Tables 6** and **7** for 2004 and 2005, respectively. Most of the compounds demonstrated a much higher percentage of one isomer over the other, particularly α-ionone, α-pinene, and the lactones, with more than 90% of one isomer. Linalool is nearly a racemic mixture, with a slightly higher percentage of the later eluting isomer. Terpinen-4-ol is an 80/20 mixture favoring the later eluting isomer.

Little variation was observed for the isomeric compounds for either year studied (**Tables 6** and **7**). There was little difference between the years, states, or varieties for isomeric ratios.

In conclusion, no flavor compounds tested in this study showed any difference between the transgenic lines and the wild type 'Meeker' raspberry.

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Received for review January 24, 2008. Revised manuscript received May 8, 2008. Accepted May 10, 2008.

JF800253E