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RESEARCH PAPER

Tracing the history of plant traits under domestication in cranberries: potential consequences on anti-herbivore defences

Cesar Rodriguez-Saona[1,*], Nicholi Vorsa[1,2], Ajay P. Singh[2], Jennifer Johnson-Cicalese[1], Zsofia Szendrei[3], Mark C. Mescher[4] and Christopher J. Frost[4,5]

[1] Phillip E Marucci Center for Blueberry and Cranberry Research and Extension, Rutgers–The State University of New Jersey, 125A Lake Oswego Rd, Chatsworth, NJ 08019, USA
[2] Department of Plant Biology and Pathology, Rutgers–The State University of New Jersey, New Brunswick, NJ 08901, USA
[3] Department of Entomology, Michigan State University, 243 Natural Sciences Building, East Lansing, MI 48824, USA
[4] Centre for Chemical Ecology and Department of Entomology, Pennsylvania State University, 501 ASI Building, University Park, PA 16802, USA
[5] Schatz Center for Tree Molecular Genetics and School of Forest Resources, Pennsylvania State University, 501 ASI Building, University Park, PA 16802, USA

* To whom correspondence should be addressed: E-mail: crodriguez@aesop.rutgers.edu

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Abstract

The process of selecting certain desirable traits for plant breeding may compromise other potentially important traits, such as defences against pests; however, specific phenotypic changes occurring over the course of domestication are unknown for most domesticated plants. Cranberry (*Vaccinium macrocarpon*) offers a unique opportunity to study such changes: its domestication occurred recently, and we have access to the wild ancestors and intermediate varieties used in past crosses. In order to investigate whether breeding for increased yield and fruit quality traits may indirectly affect anti-herbivore defences, the chemical defences have been examined of five related cranberry varieties that span the history of domestication against a common folivore, the gypsy moth (*Lymantria dispar*). Direct defences were assessed by measuring the performance of gypsy moth caterpillars and levels of phenolic compounds in leaves, and indirect defences by assaying induced leaf volatile emissions. Our results suggest that breeding in cranberry has compromised plant defences: caterpillars performed best on the derived NJS98-23 (the highest-yielding variety) and its parent Ben Lear. Moreover, NJS98-23 showed reduced induction of volatile sesquiterpenes, and had lower concentrations of the defence-related hormone *cis*-jasmonic acid (JA) than ancestral varieties. However, induced direct defences were not obviously affected by breeding, as exogenous JA applications reduced caterpillar growth and increased the amounts of phenolics independent of variety. Our results suggest that compromised chemical defences in high-yielding cranberry varieties may lead to greater herbivore damage which, in turn, may require more intensive pesticide control measures. This finding should inform the direction of future breeding programmes.

Key words: Direct and indirect defences, gypsy moth, herbivory, jasmonates, plant hormones, phenolic compounds, volatiles.

Introduction

The process of domesticating wild plant species for agriculture, which includes breeding and selection for desirable traits, can considerably alter plant phenotypes in both intended and unintended ways. Historically, the development of high-yielding plant varieties has been a priority for most crop breeders (Cock *et al.*, 1979; Bell *et al.*, 1995;

Ying *et al.*, 1998; Peng *et al.*, 1999). Breeding programmes have less often addressed plant traits relating to insect resistance (Painter, 1951; Maxwell and Jennings, 1980). Moreover, the functions of some plant defence traits—such as those mediated by the induced release of volatile organic compounds—have only been discovered relatively recently (Turlings *et al.*, 1990), and thus have not been the target of selection breeding criteria.

Many staple crop plants have been dramatically transformed from their wild ancestors and lack a sexually compatible relative species, making difficult the study of specific changes that domestication has had on particular plant traits (Gepts, 2004). Being recently domesticated, cultivated American cranberry (*Vaccinium macrocarpon* Ait.) offers an unusual opportunity to investigate these processes directly. Cranberries, perennial vines native to North America, have been domesticated for less than 160 years and farmers still use native selections. Compared with many other crop plants, cranberries are little evolved from their wild relatives, and extant populations persist in regions with domesticated varieties (Hancock *et al.*, 2008). Moreover, there is an excellent record of past selective breeding efforts in cranberry, and many of the varieties used in past breeding programmes remain extant and available. The first organized cranberry breeding programme was initiated by the USDA in 1929 (Hancock *et al.*, 2008) in response to the emergence of a serious disease, false blossom, caused by a phytoplasma vectored by the blunt-nosed leafhopper (Galleta and Ballington, 1996). Plants with reported resistance to the leafhopper were used in many early crosses, including McFarlin, Early Black, and Shaw's Success (Stevens, 1931). Subsequently, breeding criteria has shifted towards increased productivity, colour intensity, and early fruit maturation (Vorsa *et al.*, 2003; Hancock *et al.*, 2008). Taking advantage of the natural experiment provided by this history of breeding, the current study investigated the variation in plant defence characteristics of a number of varieties used in past crosses in the genetic enhancement of cranberries.

Plants produce a wide array of secondary metabolites to defend themselves against herbivores (Rosenthal and Berenbaum, 1992; Bernays and Chapman, 1994; Schoonhoven *et al.*, 1998, and references therein), and this diverse plant defence chemistry could be impacted by selective breeding. Some plant secondary metabolites directly reduce the survival, growth, and feeding of herbivores on plants; these include toxic, growth inhibitory, antifeedant, and repellent substances (Bernays and Chapman, 1994; Schoonhoven *et al.*, 1998). By contrast, indirect chemical defences reduce the performance and preference of herbivores by recruiting the herbivores' natural enemies (Dicke, 1999; Heil, 2008). A particularly important class of indirect defences entails the emission of volatiles from plants that can be used as foraging cues by predators and parasitoids (Paré and Tumlinson, 1999), and by receiving plant tissues as an alarm signal (Frost *et al.*, 2008; Rodriguez-Saona *et al.*, 2009). Since plant-derived chemicals that confer direct or indirect resistance can be costly to produce and maintain, there is considerable natural spatial and temporal variation in the expression of plant defences. Some defences are constitutively expressed, while others are induced only in response to herbivore feeding (Karban and Baldwin, 1997). Both types of defences can significantly affect herbivores. For example, Agrawal (1998) found that wild radish (*Raphanus sativus* L.) damaged by *Pieris rapae* L. caterpillars had increased concentrations of glucosinolates (as well as higher trichome densities), and these plants subsequently received less herbivory and exhibited increased reproductive fitness compared with non-induced plants. Similarly, herbivore feeding has been found to induce the emissions of volatile compounds in the majority of plant species examined to date (Turlings *et al.*, 1990, 1995; Vet and Dicke, 1992; Rodriguez-Saona *et al.*, 2009).

In order to study how selective breeding has altered direct and indirect plant defences of cranberries, the defence characteristics of five cranberry varieties, including three native selections and two varieties resulting from breeding and selection cycles, were examined in response to feeding by larvae of the common generalist folivore gypsy moth, *Lymantria dispar* (L.). In addition to assaying plant growth and the performance of feeding caterpillars directly, the levels of defence compounds (phenolics and flavonols) and phytohormones in leaves and feeding-induced volatile emissions were assayed. Plant responses to exogenous application of the defence signalling compounds jasmonic acid (JA) and methyl jasmonate (JA-Me) were also examined. Our results provide novel insights into the potential costs associated with plant breeding for high yield and other fruit quality traits on resistance to insects.

Materials and methods

Cranberry varieties and propagation

Five cranberry varieties were used: McFarlin, Potter, Stevens, Ben Lear, and NJS98-23 (Fig. 1). McFarlin is a wild selection from South Carver, MA, first cultivated in 1874, and still widely grown in the Pacific Northwest. Potter was also selected from a wild

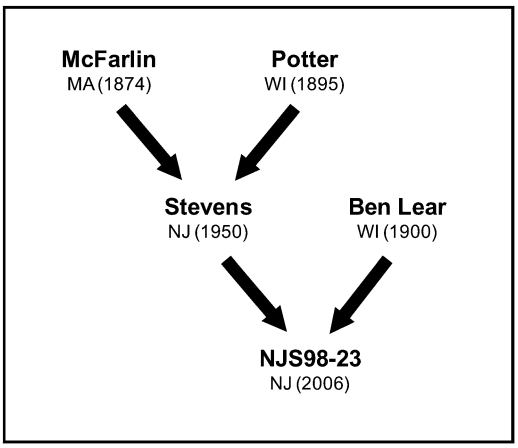

Fig. 1. History of breeding crosses among cranberry varieties used in this study. MA (Massachusetts, USA), WI (Wisconsin, USA), and NJ (New Jersey, USA) are the regions of origin, with year of first cultivation in parenthesis.

cranberry bog in Wisconsin Rapids, WI, and initially planted in 1895. McFarlin and Potter are of particular interest because they are the parents of Stevens, a variety introduced in 1950 that is currently the most widely used cranberry variety in cultivation. Stevens was produced by the initial USDA cranberry breeding programme, and was selected for high productivity, moderate red colour, and firmness (Dana, 1983). Ben Lear is a native variety originating from Berlin, WI, that was first cultivated in 1900. NJS98-23 is two generations removed from McFarlin and Potter, resulting from a cross between Stevens and Ben Lear, it was released by Rutgers University in 2006 (US Patent PP18252 2007), and was selected for early-ripening season, high fruit anthocyanin content, large fruit size, and high productivity.

Stem cuttings of all five cranberry varieties were harvested in early August 2007 from field plots belonging to the Rutgers cranberry breeding programme (PE Marucci Center for Blueberry and Cranberry Research and Extension, Chatsworth, NJ, USA). The identities of the varieties were confirmed by DNA fingerprinting using SCAR markers (Polashock and Vorsa, 2002). Five 7 cm cuttings were planted in a 50:50 v/v peat:sand mix in individual 4×4 cm cells. A total of 72 cells (360 cuttings) were planted for each variety in 52×26 cm flats. Cuttings were subjected to winter chilling in a greenhouse kept at 10±2 °C. In December, cuttings were transferred to 7 cm pots. One subset of potted plants (n=23 per variety) was used for experiments on insect performance and direct chemical defence analyses (see below). Three of five cuttings in each pot were used for insect performance assays, while the other two cuttings were used for phenolic and hormonal analyses (one cutting for each analysis). A different subset of plants (n=14 pots per variety) was used to measure volatile emissions (see below). In February 2008, pots were moved to a warm greenhouse (20±2 °C; 70±10% RH; 15:9 L:D), where they remained until they were used in experiments. Plants were then fertilized bi-weekly with PRO-SOL 20-20-20 N-P-K All Purpose Plant Food (Pro Sol Inc., Ozark, AL, USA) at a rate of 165 ppm N, and watered daily. A month after plants were transferred to a warm greenhouse, gypsy moth performance (caterpillar growth and survival), leaf chemistry (phenolic acids, flavonols, volatiles, and phytohormones), and plant growth, were measured as described below.

Insect performance

For each variety, plants (n=5 per variety per treatment) were either sprayed with 1 ml of a 1 mM solution of jasmonic acid (JA) (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.4% acetone, or 1 ml of distilled water with 0.4% acetone (untreated controls). Jasmonates, for example, JA and its volatile derivative JA-Me, induce the octadecanoid pathway in plants, resulting in the activation of direct and indirect defences against insect herbivores (Karban and Baldwin, 1997; Walling, 2000; de Bruxelles and Roberts, 2001). Jasmonates are widely used to test the induction of anti-herbivore defences in plants (Thaler, 1999; Rodriguez-Saona *et al.*, 2001; Thaler *et al.*, 2001, 2002). Treatments were applied at 08.00 h using a 60 ml spray bottle (Setco Inc., Cranbury, NJ, USA). Four hours after treatment, three uprights per pot were inserted in florists' water picks, enclosed in a ventilated 40 dram plastic vial, and secured on Styrofoam trays. The following day, one neonate gypsy moth was placed in each vial. Neonates were used because the performance of early instar gypsy moths is strongly influenced by host plant chemistry (Beninger and Abou-Zaid, 1997). Gypsy moth caterpillars were obtained from a laboratory colony maintained at the Rutgers Marucci Center, and reared on a wheat germ diet at 24±1 °C, 65% RH, and 14:10 L:D. Plants and insects were kept in the greenhouse, under the conditions described above, throughout the experiment. Caterpillar mortality and mass were assessed 7 d after transfer (missing caterpillars were assumed to be dead). The experiment was replicated three times (n=15 per variety).

To confirm that the effects of the JA treatment on caterpillar growth were the result of plant effects rather than any direct toxic effects of JA, an additional experiment was conducted to test the toxicity of JA to gypsy moth neonates. Individual neonate gypsy moth caterpillars were placed in 30 ml plastic cups containing ~10 ml of wheat germ diet. The diet in each cup was sprayed with either 1 mM JA solution with 0.4% acetone, distilled water with 0.4% acetone, or distilled water alone 4 h prior to placing the caterpillars (n=20 cups per treatment). Caterpillar mortality and mass were recorded after 7 d, and the experiment was replicated twice.

Leaf chemistry

Phenolic analysis: Flavonols and phenolic acids are ubiquitous compounds in plants that often play important roles in resistance against insects (Bernays and Chapman, 1994; Simmonds, 2001). Leaf flavonols (quercetin glycosides) and phenolic acids from JA-treated and untreated plants were measured for each cranberry variety (n=3 per variety per treatment). The JA treatment followed the methods described above. After treatment, leaves were frozen in liquid nitrogen and kept at –20 °C before extraction. Frozen leaves were macerated in liquid nitrogen using a mortar and pestle. Ground leaf tissue (30 mg) was placed in 2.0 ml Eppendorf tubes containing 1 ml of 50% methanol:25% acetone:24% water (by vol.) and 1% formic acid. Extraction was performed using vortex for ~30 s followed by sonication for 10 min. Samples were centrifuged at 13 000 rpm for 10 min and the supernatant was collected in separate Eppendorf tubes. Extraction was performed one more time with 500 µl of solvent as described above. Both supernatants were mixed together and filtered using Spin-X microcentrifuge filters at 5 000 rpm for 0.5 min.

Filtered extracts were subjected to HPLC and LC-MS-MS analysis. Leaf flavonol (quercetin glycosides) analysis was performed using a Dionex liquid chromatography system with a Series AS-50 autosampler, GP-40 gradient pump, AS50 thermal compartment, PDA-100 photo diode array detector (Dionex Corporation, Sunnyvale, CA, USA). The HPLC system (Dionex Corporation, IL,USA) employed a Hypersil Gold C18 (3 µm particle size; 150 mm length×3.0 mm ID; Thermo Electron Co., Bellefonte, PA, USA). Five µl was injected onto the column and a gradient elution was used for separations. Solvent A consisted of 10% MeOH in H_2O adjusted to pH 3.5 with formic acid. Solvent B consisted of 20% H_2O (pH 3.5), 20% MeOH, and 60% acetonitrile. At a flow rate of 0.3 ml min^{-1}, the following gradient was used: 0 min, 100% A; 10 min, 40% A; 20 min, 20% A; 40 min, 0% A; held at 0% A for 15 min. Five minutes of equilibration at 100% A was performed before and after each injection. The column outlet was coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada) equipped with an electrospray ionization (ESI) source. Leaf flavonol analysis was performed by ESI with ion spray voltage at 4500 V and temperature at 350 °C. The instrument operated in a negative ion mode. For full-scan HPLC-ESI-MS analysis, spectra were scanned in the range of 50–1200 m/z. Data acquisition and processing were performed using an Analyst 1.4.2 data system (Applied Biosystems, Foster City, CA, USA).

The identification of quercetin glycosides was made by comparing retention times, UV spectral patterns, and ESI-MS fragmentation patterns with authentic standards (Indofine Chemical Company, Inc., Somerville, NJ, USA) and published data. Quercetin derivatives were quantified using the corresponding authentic standard for each compound. Quantification of phenolic acids was based on a standard curve prepared with 5-caffeoylquinic acid. For both the standards and extracts, 5 µl were injected and separated using the aforementioned column and solvent system, but at a flow rate of 1 ml min^{-1}. Scanning by the PDA was at 325 nm and 366 nm.

Volatile collections and analysis

Two experiments were conducted to determine the volatile response of cranberry leaves to gypsy moth feeding and exogenous

JA-Me treatment. In the first experiment, volatiles were collected from plants either damaged by four gypsy moth caterpillars (2nd instars) or left undamaged. Caterpillars in the damage treatment were allowed to feed on plants for 2 d prior to volatile collection, and remained on the plants during volatile collections; gypsy moth caterpillars themselves, as well as their frass are undetectable by the volatile collection methods employed (Staudt and Lhoutellier, 2007; Rodriguez-Saona *et al.*, 2009). After volatile collection, the amount of damage was estimated by counting the number of leaves within each terminal that had feeding damage; all leaves from the plants were then collected, oven-dried, and weighed. The entire experiment was replicated four times (n=4 per variety per treatment). In a separate experiment, volatiles were collected from the five cranberry varieties treated with 2 ml of either 1 mM JA-Me (Sigma-Aldrich) in a 0.1% Tween-20 solution, or 2 ml of a 0.1% Tween-20 solution (control). JA-Me was applied the day prior to volatile collections (17.00 h). The experiment was replicated three times (n=3 per variety per treatment).

Volatile emissions were sampled in the greenhouse using a pull system (Tholl and Röse, 2006). The above-ground portion of five cranberry terminals were enclosed inside a 20×20 cm volatile collection bag made of non-absorbent Vac-Pak material (Richmond Products, Norwalk, CA, USA). Binder clips were used to close the bag opening around the stem. Volatiles from inside the bag were collected in 30 mg Super-Q adsorbent traps (Alltech, Deerfield, IL, USA) by pulling air at a rate of 600 ml min^{-1} with the aid of a 12 V vacuum pump (Sensidyne, Clearwater, FL, USA). Volatiles were collected for 3 h (11.00–14.00 h). Empty bags were sampled concurrently to test for contamination. After collection, all terminals from plants were harvested, oven-dried at 60 °C, and weighed, and the bags rinsed with tap water and 70% ethanol.

The collected volatiles from Super-Q traps were eluted with dichloromethane (150 µl) containing 400 ng of n-octane (Sigma-Aldrich) as internal standard (IS). Samples were analysed on a Hewlett Packard 6890 Series Gas Chromatograph (GC) equipped with a flame ionization detector. The program for separation and quantification (Agilent HP-1 column: 10 m × 0.53 mm×2.65 µm, He as carrier gas: constant flow=5 ml min^{-1}, velocity=39 cm s^{-1}) was 40 °C initial temperature (1 min), 14 °C min^{-1} to 180 °C (2 min), then 40 °C min^{-1} to 200 °C, then 200 °C (2 min). Compounds (ng h^{-1}) were quantified based on comparison of peak areas with that of the IS (n-octane). Identification of compounds was performed on a Varian 3400 GC coupled to a Finnigan MAT 8230 mass spectrometer (MS). The program (Supelco MDN-5S column: 30 m×0.32 mm×0.25 µm) was 35 °C initial temperature (1 min), 4 °C min^{-1} to 170 °C, then 15 °C min^{-1} to 280 °C. The MS data were acquired and processed in a Finnigan MAT SS300 data system, and compounds were identified by GC retention index, and comparison of their retention times to those of commercially available compounds and their spectral data to those from the NIST library (Rodriguez-Saona *et al.*, 2009).

Hormone analysis

Two separate experiments were conducted to assess the effects of induction on defence hormones in cranberry leaves. The acidic phytohormones JA and salicylic acid (SA), and their acidic precursors [linolenic acid (LNA) and linoleic acid (LA) for JA, cinnamic acid (CA) for SA] were measured. JA and SA are important signalling molecules for different plant defence responses. In the first experiment, JA, LA, LNA, CA, and SA from plants damaged by gypsy moth caterpillars were measured. One cranberry terminal per pot was bagged with a spun polyester sleeve (Rockingham Opportunities Corp., Reidsville, NC, USA). For the insect-damage treatment, one 3rd instar gypsy moth was placed inside the bags and allowed to feed on plants for 2 d. For the controls, plants were also bagged but received no gypsy moth (n=4 per variety per treatment). In the second experiment, LNA, LA, SA, and CA were measured from JA-treated and untreated plants for each cranberry variety (n=5 per variety per treatment). Endogenous JA was not measured in this experiment because of the confounding effects of treatment. The JA treatment was conducted as described above.

After treatment, cranberry terminals were frozen in liquid nitrogen and kept at –20 °C before extraction. Extraction and quantification of phytohormones were performed as previously described (Frost *et al.*, 2008). Briefly, ~100 mg frozen leaf tissue of each sample was ground with a mortar and pestle, and transferred to FastPrep tubes (Qbiogene, Carlsbad, CA, USA) containing Zirmil beads (SEPR Ceramic Beads and Powders, Mountainside, NJ, USA). Dihydro-JA, $^{2}H_{6}$-SA, gamma-LNA, and d5-CA (CDN Isotopes, Pointe-Claire, Quebec, Canada) were added as IS sample addition (100 ng each). The samples were mixed with 1-propanol:-H_2O:HCl (2:1:0.002, by vol.) and homogenized in a FastPrep FP 120 (Qbiogene). Dichloromethane was quickly added to each sample, which was shaken and centrifuged. The bottom organic phase (dichloromethane) was transferred to a glass screw-cap vial and evaporated by a constant airstream. Each sample was reconstituted in diethyl ether:methanol (9:1, v/v), and carboxylic acids were converted into methyl esters using a trimethylsilyldiazomethane (Sigma-Aldrich). Volatile metabolites were separated from the complex mixture by vapour-phase extraction and analysed by chemical ionization-GC-MS as previously described (Schmelz *et al.*, 2003, 2004).

Plant growth patterns

During the course of our experiments apparent differences among varieties in the size of plants were noted. To quantify growth differences among varieties, the size of 10 randomly selected plants from each variety were measured by obtaining stolon and shoot lengths and counting the total number of vines (total growth=sum length of all vines). The length of a fully-expanded leaf was also measured for each of the 10 plants from each variety.

Statistical analyses

Prior to analyses, data on caterpillar survival and mass were averaged per pot. Data were analysed using 2-way ANOVA (Minitab 13, Minitab Inc., State College, PA, USA) to determine the effects of variety, JA treatment, and their interaction on caterpillar mass and mortality, followed by means separation by Tukey test (α=0.05). Caterpillar masses were $\log_{10}$-transformed and per cent data arcsine square-root transformed prior to analyses. Differences in plant size (total growth and lengths of vines and leaves) among varieties were analysed using ANOVA, followed by means separation by Tukey tests. Where needed, plant size data were $\log_{10}$-transformed prior to analyses.

Data on leaf chemistry were analysed using 2-way multivariate analysis of variance (MANOVA; Minitab) with variety, treatment (JA or gypsy moth treatment versus controls), and their interaction as sources of variation. Separate MANOVAs were conducted for each chemical group, i.e. flavonols, phenolic acids, volatiles, and hormones. Volatiles were classified based on their biosynthetic origin into alcohols, esters, benzenoids, monoterpenes, and sesquiterpenes, and analysed as groups using MANOVA. A significant effect was followed by ANOVA to determine which specific compounds within groups varied among varieties; if significant, multiple comparison Tukey tests were used to determine differences among means (α=0.05). In addition, a principal component analysis was performed on the groups of measured leaf chemicals and correlated with gypsy moth caterpillar mass (R Statistical Software, 2008). Pearson correlations (Minitab) were conducted to correlate levels of individual chemicals within groups with caterpillar mass and chemicals between groups.

Results

Insect performance

Cranberry varieties varied in their constitutive direct resistance against gypsy moth caterpillars (F=26.69; df=4, 121; P <0.01). Caterpillar mass was higher on Ben Lear and NJS98-23 compared with McFarlin, Potter, and Stevens (Fig. 2A). By contrast, induced direct resistance was not affected by variety: JA treatment reduced caterpillar mass by 23% (F=5.12; df=1, 121; P=0.03) independent of variety (F=0.36; df=4, 121; P=0.84). However, there were no variety (F=1.39; df=4, 140; P=0.24), JA treatment (F=1.05; df=4, 140; P=0.31), or variety×JA (F=0.49; df=4, 140; P=0.74) effects on gypsy moth caterpillar mortality (Fig. 2B). Further, the negative effects of JA on caterpillar mass were attributable to the activation of plant defences by JA and not to direct toxicity because caterpillar mass was similar on JA-treated and untreated diets: mean caterpillar mass (mg) ±SE on (i) an untreated diet = 2.18±0.15; (ii) on a JA-treated diet=2.53±0.23; (iii) on an acetone-treated diet=2.39±0.15; F=0.99; df=2, 116; P=0.37). Only 1 out of 40 caterpillars (2.5%) died on the JA-treated diet, while no caterpillars died on the other treatments.

Leaf chemistry

Phenolics: Flavonol levels were affected by JA treatment and variety independently (Table 1A). Across all varieties, JA treatment increased levels of the flavonol quercetin-3-β-galactoside by ~30% compared with controls (F=5.15; df=1, 20; P=0.03) (Fig. 2C). No other flavonols were affected by JA (all P values >0.05). McFarlin had higher concentrations of quercetin-3-β-galactoside compared with Potter (F=3.34; df=4, 20; P=0.03) (Fig. 2C), and quercetin-3-α-arabinopyranoside compared with Potter and NJS98-23 (F=4.46; df=4, 20; P=0.01) (Fig. 2D). Despite these differences, there was no significant correlation between caterpillar mass and total flavonol concentrations (r^2= –0.15; P=0.69) or any single flavonol (all P values >0.05),

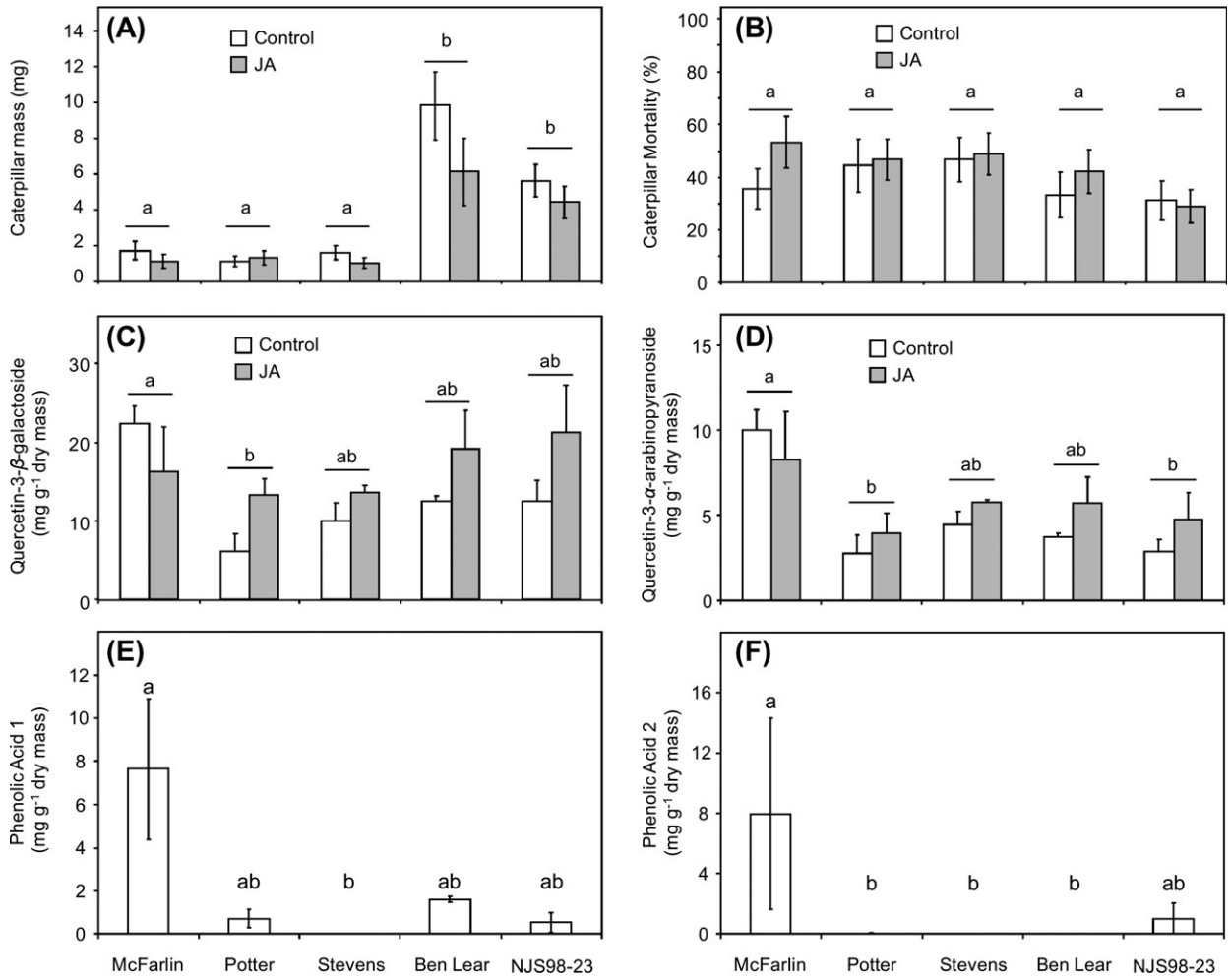

Fig. 2. Effects of cranberry varieties and exogenous jasmonic acid (JA) treatment on mass (A) and mortality (B) of 1st instar gypsy moth caterpillars after 7 d of feeding, and on the flavonols quercetin-3-β-galactoside (C) and quercetin-3-α-arabinopyranoside (D); and, effects of variety on two unknown phenolic acids, phenolic acid 1 (E) and phenolic acid 2 (F). JA-treated plants were sprayed with a 1 mM solution dissolved in 0.4% acetone. Bars indicate means ±1 SE. Different letters above the bars indicate significant differences among varieties ($P \leq 0.05$); n=15 for parameters on insect performance, n=3 for leaf chemistry analyses.

Table 1. Results of MANOVA for the effects of jasmonates (jasmonic acid or methyl jasmonate) or gypsy moth feeding and variety on cranberry chemistry

Chemical class	Treatment			Variety			Treatment×Variety		
	Wilks' λ	*F*	*P*	Wilks' λ	*F*	*P*	Wilks' λ	*F*	*P*
(A) Jasmonate application[a]									
Flavonols[b]	0.51	3.08	0.04	0.10	2.66	0.00	0.26	1.36	0.18
Phenolic acids[c]	0.43	1.75	0.18	0.03	2.08	0.01	0.15	0.85	0.69
Volatiles									
Alcohols[d]	0.10	81.94	< 0.01	0.60	1.38	0.24	0.84	0.44	0.89
Esters[d]	0.02	394.86	< 0.01	0.61	0.82	0.63	0.57	0.95	0.51
Benzenoids[d,e]	0.24	18.56	< 0.01	0.65	0.72	0.73	0.75	0.45	0.93
Monoterpenes[d]	0.04	28.83	< 0.01	0.04	1.44	0.12	0.15	0.71	0.86
Sesquiterpenes[d]	< 0.01	674.18	< 0.01	0.11	1.06	0.43	0.12	0.97	0.54
Phytohormones[f]	0.70	3.90	0.01	0.41	2.45	0.00	0.77	0.63	0.85
(B) Gypsy moth feeding									
Volatiles									
Alcohols	0.24	45.14	<0.01	0.61	2.02	0.06	0.65	1.77	0.10
Esters	0.47	7.71	<0.01	0.56	1.07	0.39	0.62	0.88	0.59
Benzenoids	0.26	25.98	<0.01	0.76	0.70	0.75	0.73	0.78	0.67
Monoterpenes	0.07	26.21	<0.01	0.14	1.36	0.12	0.15	1.31	0.15
Sesquiterpenes	0.10	23.32	<0.01	0.04	3.05	<0.01	0.04	3.05	<0.01
Phytohormones[g]	0.42	5.84	<0.01	0.15	2.62	<0.01	0.40	1.12	0.34

[a] Jasmonic acid (JA) was used in phenolic and hormonal analyses, Methyl jasmonate (JA-Me) was used in volatile analyses.
[b] Flavonols include quercetin-3-β-galactoside, quercetin-3-xylopyranoside, quercetin-3-α-arabinopyranoside, quercetin-3-α-arabinofuranoside, and quercetin-3-rhamnopyranoside; df, error df: 1, 20 (Treatment), 4, 20 (Variety), 4, 20 (Treatment×Variety).
[c] Phenolic acids include nine compounds of unknown identity; df, error df: 1, 20 (Treatment), 4, 20 (Variety), 4, 20 (Treatment×Variety).
[d] See Table 2 for a list of alcohols, esters, benzenoids, monoterpenes, and sequiterpenes; df, error df: jasmonates=1, 20 (Treatment), 4, 20 (Variety), 4, 20 (Treatment×Variety); gypsy moth=1, 30 (Treatment), 4, 30 (Variety), 4, 30 (Treatment×Variety).
[e] Products of the shikimic acid pathway.
[f] Phytohormones include salicylic acid (SA), and the precursor acids linolenic acid (LNA), linoleic acid (LA), and cinnamic acid (CA); df, error df: 1, 40 (Treatment), 4, 40 (Variety), 4, 40 (Treatment×Variety).
[g] Phytohormones include *trans*-JA, *cis*-JA, and SA, and their precursors LNA, LA, and CA; df, error df: 1, 30 (Treatment), 4, 30 (Variety), 4, 30 (Treatment×Variety).

indicating that flavonol concentrations alone could not explain gypsy moth resistance in cranberries.

Phenolic acid concentrations were also affected by variety, but not by JA treatment or JA × variety interaction (Table 1A). The concentrations of two of the nine phenolic acids detected in our analyses (all of unknown identity) differed among varieties (Fig. 2E, F). McFarlin had greater amounts of both compounds compared with all other varieties (compound 1: F=4.98, df=4, 10, P=0.02, Fig. 2E; compound 2: F=6.19, df=4, 10, P <0.01, Fig. 2F). Similar to flavonols, total phenolic acid levels (r^2=0.25; P=0.55), or individual phenolic acids (all P values >0.05), were not correlated with caterpillar mass.

Volatiles: The two most derived cranberry varieties (Stevens and NJS98-23) showed significantly lower induced volatile emissions following gypsy moth feeding compared with the parental cranberry varieties. As expected, constitutive volatile emissions were low for all varieties (Fig. 3), and gypsy moth feeding significantly increased volatile emissions compared with the controls [mean volatile emissions (ng g^{-1} h^{-1}) ±SE: control=164.1±19.0; gypsy moth treatment=969±144; F=82.6; F=82.6; df=1, 38; P <0.01] (Table 1B). The compounds induced by gypsy moth feeding included a complex blend of alcohols, esters, benzenoids, monoterpenes, and sesquiterpenes (Table 2). The induced release of sesquiterpenes, in particular, varied by variety (Table 1B): gypsy moth feeding induced emissions of various sesquiterpenes in McFarlin, Potter, and Ben Lear, whereas Stevens and NJS98-23 lacked this response (Fig. 3A–C). In fact, total amounts of sesquiterpenes in NJS98-23 were the same on both control plants and those fed upon by gypsy moth caterpillars (P >0.05) (Fig. 3D). That is, NJS98-23 did not induce sesquiterpene emissions in response to herbivory. This effect was not the result of differences in insect feeding among varieties [per cent number of damaged leaves (mean ±SE)=29±0.03%; F=0.4; df=1, 15; P=0.80], or in the amount of tissue sampled for volatile collections [mean plant dry mass (mean ±SE)=1.4±0.2 g; F=0.46; df=4, 25; P=0.76].

JA-Me treatment induced qualitatively similar volatile emissions to those induced by gypsy moth feeding (Table 2), but variety was not a factor quantitatively (Table 1A). Total volatile emissions significantly increased in the JA-Me treatment compared with the controls (mean volatile emissions ±SE: control=290.9±32.5; JA-Me treatment=11500.4±976.7; F=492.2; df=1, 28; P <0.01).

Hormones: Since JA signalling is often critical in regulating induced defences, phytohormones in leaves fed upon

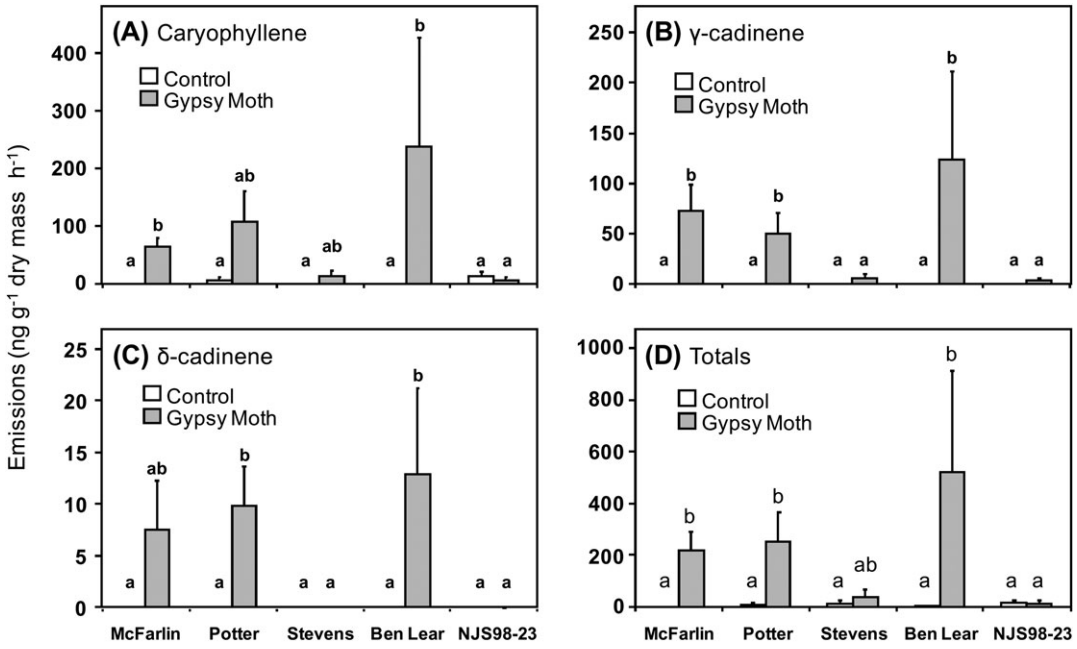

Fig. 3. Means ±1 SE of caryophyllene (A), γ-cadinene (B), δ-cadinene (C), and total sesquiterpenes (D) in different cranberry varieties and between control and gypsy moth treatments. For the gypsy moth treatment, four 2nd instar gypsy moth caterpillars were allowed to feed on plants for 2 d prior to volatile collections, and remained on the plants during volatile collections. Control treatment received no insect feeding. Volatiles were collected for 3 h (11.00–14.00 h). Different letters above the bars indicate significant differences between treatments and among varieties ($P \leqslant 0.05$); $n=4$.

by gypsy moth caterpillars were measured. On average, gypsy moth damaged plants had ~6 times higher levels of *cis*-JA compared with undamaged plants ($F=26.5$; df=1, 30; $P<0.01$). The induction of *cis*-JA by gypsy moth feeding also varied by variety: NJS98-23 had significantly lower amounts of *cis*-JA than McFarlin or Ben Lear ($F=3.2$; df=4, 30; $P=0.03$) (Table 1B; Fig. 4), and the lowest amounts of LA compared with the other varieties ($F=2.6$; df=4, 30; $P=0.05$). Despite this, there was no significant interaction between gypsy moth feeding and variety on *cis*-JA or LA concentrations (*cis*-JA: $F=0.44$; df=4, 30; $P=0.78$; LA: $F=2.11$; df=4, 30; $P=0.10$). There were no effects of insect feeding, variety, or their interaction on other hormones and fatty acids (e.g. CA, LNA, SA; all P values >0.05). There was a significant negative correlation between the plant acidic hormones measured and gypsy moth mass ($r^2= -0.64$; $P=0.05$). Interestingly, SA most strongly correlated with caterpillar mass (Pearson, $r= -0.62$; $P=0.06$). There was also a strong positive correlation between *cis*-JA and the flavonol quercetin-3-α-arabinopyranoside (Pearson, $r=0.94$; $P=0.02$; also compare patterns on Figs 2D and 4).

When the effects of exogenous JA application were examined, JA treatment significantly altered plant defence-related hormones independently of variety (Table 1A). JA increased levels of LNA by 25% ($F=6.9$; df=1, 40; $P=0.01$). No effects of JA applications were detected for LA, CA, and SA (all P values >0.05). Variety had a significant effect on LA ($F=2.89$; df=4, 40; $P=0.03$) and CA ($F=3.26$; df=4, 40; $P=0.02$), but not on LNA or SA ($P>0.05$). Stevens had 31% lower LA and 2-fold higher CA concentrations than Potter and Ben Lear, respectively ($P \leqslant 0.05$). Measuring endogenous JA concentrations was not possible on JA-treated plants.

Plant growth patterns

Potter and NJS98-23 had the greatest total growth while Ben Lear had the least ($F=3.67$; df=4, 45; $P=0.01$) (Fig. 5A). However, individual vines in McFarlin were the longest while Potter and Ben Lear vines were the shortest ($F=3.97$; df=4, 45; $P<0.01$) (Fig. 5B). Potter leaves were the shortest while those of McFarlin and NJS98-23 were longest ($F=3.76$; df=4, 45; $P=0.01$) (Fig. 5C). These results indicate significant variation in the growth pattern among varieties: (i) McFarlin and NJS98-23 were the biggest plants based on leaf length and individual and total vine length; (ii) Potter and Ben Lear both had the shortest vines but Potter had more of them; and (iii) Potter had the smallest leaves.

Discussion

Intentional and unintentional changes in plant phenotypes resulting from domestication and selective breeding can have profound and unexpected consequences on interactions involving plants, herbivores, and natural enemies (Benrey *et al.*, 1998; Chen and Welter, 2005, 2007; Wang *et al.*, 2009). Because of the historical focus on breeding for high yield and specific fruit traits, for example, early-season ripening, colour intensity, and size, breeding programmes

Table 2. Results of ANOVA for the effects of gypsy moth feeding or methyl jasmonate (JA-Me) treatment on cranberry volatile emissions

Chemical	Gypsy moth[a]				JA-Me[a]			
	F	df	error df	*P*	*F*	df	error df	*P*
Alcohols								
Hexanol	10.61	1	30	<0.01	129.60	1	20	<0.01
(*Z*)-3-hexen-1-ol	90.20	1	30	<0.01	149.90	1	20	<0.01
Esters								
(*Z*)-3-hexenyl acetate	11.26	1	30	<0.01	12.00	1	20	<0.01
(*Z*)-3-hexenyl butyrate	10.26	1	30	<0.01	40.30	1	20	<0.01
Phenylethyl acetate	23.55	1	30	<0.01	1103.50	1	20	<0.01
Benzenoids								
Benzene acetonitrile	16.50	1	30	<0.01	11.60	1	20	<0.01
Indole	64.70	1	30	<0.01	52.80	1	20	<0.01
Monoterpenes								
α-Pinene	0.30	1	30	0.58	0.60	1	20	0.62
Camphene	93.00	1	30	<0.01	49.60	1	20	<0.01
β-Pinene	18.60	1	30	<0.01	28.30	1	20	<0.01
Myrcene	76.50	1	30	<0.01	165.20	1	20	<0.01
Limonene	30.10	1	30	<0.01	18.10	1	20	<0.01
Eucalyptol	52.40	1	30	<0.01	29.30	1	20	<0.01
Linalool oxide	1.80	1	30	0.19	0.10	1	20	0.97
Linalool	35.80	1	30	<0.01	135.60	1	20	<0.01
Myrcenone	208.80	1	30	<0.01	98.60	1	20	<0.01
α-Terpineol	1.90	1	30	0.18	6.04	1	20	0.02
Sesquiterpenes								
α-Cubebene	2.70	1	30	0.11	24.40	1	20	<0.01
Copaene	7.80	1	30	<0.01	127.00	1	20	<0.01
Muurolene	17.50	1	30	<0.01	3934.60	1	20	<0.01
Caryophyllene	26.50	1	30	<0.01	144.80	1	20	<0.01
β-Farnesene	1.90	1	30	0.17	148.20	1	20	<0.01
Humulene	34.60	1	30	<0.01	385.00	1	20	<0.01
γ-Cadinene	112.90	1	30	<0.01	110.30	1	20	<0.01
δ-Cadinene	18.30	1	30	<0.01	420.30	1	20	<0.01
α-Farnesene	3.90	1	30	0.06	72.80	1	20	<0.01

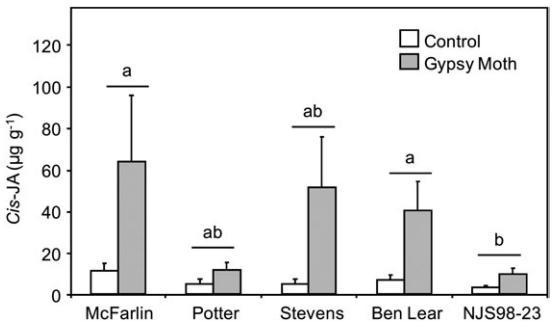

Fig. 4. Means ±1 SE of *cis*-jasmonic acid (JA) in different cranberry varieties and between control and gypsy moth treatments. For the gypsy moth treatment, a single cranberry terminal was damaged by a 3rd instar gypsy moth caterpillar for 2 d prior to analysis. Control treatment received no gypsy moth. Different letters above the bars indicate significant differences between treatments and among varieties ($P \leq 0.05$); $n=4$.

may have compromised plant resistance against insects (Krischik and Denno, 1983; Zangerl and Bazzaz, 1992). For instance, selection for a particular horticultural trait may impact direct and indirect chemical defence traits, particularly due to linkage or pleiotropic effects. This hypothesis is grounded in the observation that plants must allocate limited resources among multiple processes, for example, reproduction and defence (Bazzaz *et al.*, 1987), and that trade-offs occur when resources are diverted from one process to another (Herms and Mattson, 1991). Thus, plants that allocate greater resources to reproduction might be less capable of defending themselves against herbivores (Obeso, 2002). However, some desirable traits favoured during domestication—such as anthocyanin-based pigmentation, which is valued for both colour and antioxidant properties—can incidentally enhance plant defences. Moreover, the effects of selective breeding on plants' anti-herbivore defences and insect resistance are uncertain, and have seldom been investigated empirically (Wink, 1988).

Our data support the hypothesis that breeding for high yield and other fruit-related traits, such as early-season ripening, colour intensity, and size, in cranberries may compromise defences against folivorous insects, though the effects of breeding varied for specific plant defence traits.

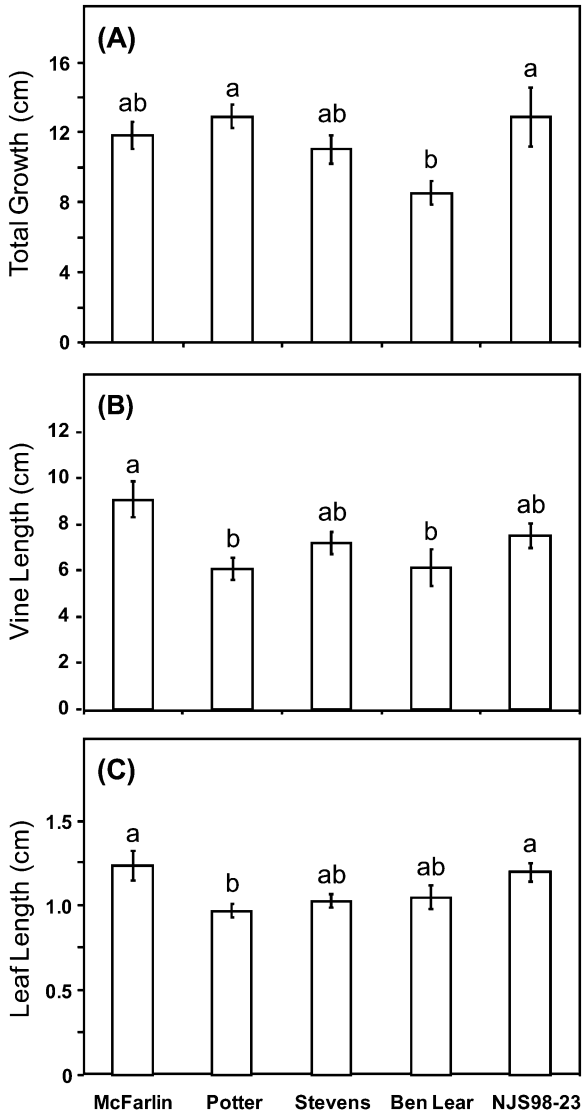

Fig. 5. Mean ±1 SE differences in total growth (A), vine length (B), and leaf length (C) among cranberry varieties. Different letters above bars indicate significant differences among varieties ($P \leq 0.05$); $n=10$.

Specifically, increased herbivore performance was observed together with a strong reduction in induced volatile emissions in the most derived cranberry variety NJS98-23. These results suggest that selective breeding focused on reproductive and fruit quality traits in cranberries may result in varieties having greater susceptibility to herbivore damage, possibly necessitating intensive pesticide controls in cultivated cranberry systems that employ it.

The substantial variability among cranberry varieties in constitutive resistance against gypsy moth caterpillars observed here provides an insight into the genetics of direct insect resistance in cranberries. In no-choice assays, gypsy moth caterpillars grew larger on the more-derived NJS98-23, as well as on Ben Lear, than on McFarlin, Potter, and Stevens. NJS98-23 and Ben Lear are both early flowering and early ripening relative to the other three varieties and may suggest their response by gypsy moth may be associated with plant phenology. Crosses between the two resistant cranberry varieties McFarlin and Potter resulted in similar levels of insect resistance in their offspring Stevens. However, this resistance was lost when Stevens was crossed with the susceptible Ben Lear, yielding the susceptible NJS98-23. Thus, a single breeding event diminished resistance against gypsy moth in cranberries, which suggests that a key component of constitutive insect resistance in cranberries is a recessive trait.

The phenolic composition of cranberry varieties may provide a plausible mechanism for constitutive resistance against gypsy moth caterpillars. For instance, Beninger and Abou-Zaid (1997) reported strong growth inhibitory activity and high gypsy moth caterpillar mortality when mixing quercetin-3-*O*-glucoside with an artificial diet. Two other quercetin glycosides, quercetin-3-β-galactoside and quercetin-3-α-arabinopyranoside, as well as several phenolic acids were present in high concentration in McFarlin and could partially explain resistance in this variety against gypsy moth caterpillars. Compared with McFarlin, the susceptible NJS98-23 had reduced amounts of quercetin-3-α-arabinopyranoside. However, the also-resistant variety Potter had low quantities of this and other phenolic compounds. As a result, caterpillar performance could not be directly linked to phenolic concentrations; rather, insect resistance in cranberries is probably due to multiple mechanisms that vary among varieties. In fact, resistance to insects can also be due to physical characteristics such as leaf toughness (Coley, 1983). Potter has smaller leaves than McFarlin, though it remains unknown whether leaf size confers resistance against gypsy moth caterpillars.

While cranberry breeding appears to have lowered direct constitutive resistance against gypsy moth caterpillars, the ability of the different varieties to induce enhanced resistance did not differ among the varieties examined. A similar, albeit weak, induction of direct resistance by the exogenous applications of JA was found for all cranberry varieties. The relatively weak contribution to insect resistance of induced defences suggests that direct constitutive resistance is paramount in cranberry. Moreover, the similar induction of direct defences among resistant and susceptible cranberry varieties suggests that limited or no trade-offs exist between constitutive and inducible direct defences. Such trade-offs have been reported in other systems; for example, Gianoli (2002) reported trade-offs between constitutive and aphid-induced defences in wheat. A lack of trade-off between induced and constitutive resistance in our study is, however, not surprising given that the induced response was weak in all cranberry genotypes, and there can be no trade-off if the requisite variation is lacking.

In contrast to direct defences, breeding history in the cranberry lines studied here has significantly lowered the inducibility of volatile compounds that have previously been implicated in indirect defence. All the varieties tested in this study emitted low amounts of volatiles constitutively, and both gypsy moth feeding and exogenous JA-Me application induced volatile emissions in all of the cranberry

varieties. Compared with JA-Me, however, gypsy moth feeding induced sesquiterpene emissions that differed among varieties: both Stevens and NJS98-23 emitted lower amounts of inducible sesquiterpenes compared with all three wild varieties McFarlin, Potter, and Ben Lear. This result is consistent with some previous work on the relationship between domestication and plant volatile emissions. For example, Loughrin *et al.* (1995) found that herbivore-induced volatile emissions from a naturalized cotton variety were seven times higher than those from commercial varieties. In particular, it was found that (*E*)-β-caryophyllene, a sesquiterpene known to attract insect parasitoids (Sasso *et al.*, 2009), lacewings (Flint *et al.*, 1979), and ladybeetles (Verheggen *et al.*, 2007), was not significantly induced in gypsy moth-damaged NJS98-23 plants. Genotypic variation in inducible (*E*)-β-caryophyllene emissions has also been reported for maize (Gouinguené *et al.*, 2001), where European varieties emit greater quantities of this volatile than North American varieties (Degen *et al.*, 2004). Such changes in volatile emissions as the result of crop domestication can disrupt tritrophic interactions by favouring the success of the herbivore and reduce the efficiency of natural enemies in some systems (Chen and Welter, 2005, 2007; Wang *et al.*, 2009). However, such interactions may actually be enhanced in other systems. For example, Benrey *et al.* (1998) found that performances of two herbivores, *Pieris rapae* L. and *Zabrotes subfasciatus* (Boheman), and their parasitoids *Cotesia glomerata* (L.) and *Stenocorse bruchivora* (Crawford), respectively, were higher on cultivated plants than on their wild relatives.

Both direct and indirect inducible defences are coordinated by signalling phytohormones, with JA presumably of central importance in response to chewing insect herbivores (Thaler *et al.*, 2002*b*). Gypsy moth feeding induced *cis*-JA in cranberries, and this induction varied among varieties, although it only partially explained the observed differences in defence profiles. Flavonols correlated with amounts of *cis*-JA, though they only minimally influenced resistance against gypsy moth. With respect to volatile emissions, McFarlin and Ben Lear emitted high amounts of inducible sesquiterpenes and had high amounts of inducible *cis*-JA; by contrast, NJS98-23 emitted low amounts of sesquiterpenes and had low amounts of inducible *cis*-JA. However, in Potter and Stevens, sesquiterpene emissions did not correlate with *cis*-JA levels. Caterpillar feeding often activates multiple defensive signalling pathways in plants, including JA- and SA-dependent pathways (Diezel *et al.*, 2009), and these may interact with each other often antagonistically (Peña-Cortés *et al.*, 1993; Doares *et al.*, 1995; Thaler *et al.*, 2002*a*). Our results, however, did not show any effects of gypsy moth feeding on SA; thus, there is no evidence that the differences in sesquiterpene emissions between gypsy moth feeding and Me-JA were due to activation of the SA-dependent pathway. The involvement of other herbivore-inducible phytohormones known also to regulate JA responses in plants, such as ethylene and abscisic acid (Kahl *et al.*, 2000; Schmelz *et al.*, 2003; Diezel *et al.*, 2009), remains unknown.

The simultaneous deployment of multiple defences can entail high fitness costs for plants (Ballhorn *et al.*, 2008; reviewed by Koricheva *et al.*, 2004). The cranberry varieties evaluated here had high levels of constitutive direct defences relative to the low levels of constitutive volatile compounds. By contrast, volatiles (indirect defences) were highly inducible, whereas direct defences were only weakly inducible. Although the role of plant volatiles as an indirect defence needs to be confirmed, our results suggest a possible trade-off between direct and indirect defences in cranberries, where resistant cranberry varieties seem to rely on elevated direct defences at all times whereas indirect defences are employed only when plants are under herbivore attack. Breeding in cranberries, however, appears to have led to varied effects on plant defences. It resulted in lower constitutive direct defences in NJS98-23, as measured by greater gypsy moth caterpillar mass and lower levels of several phenolic compounds, compared with McFarlin, one of its ancestral parents. While induced direct defences were not affected by breeding, NJS98-23 also had lower inducible sesquiterpene emissions compared with McFarlin. Since sesquiterpenes play a key role in natural enemy attraction (Flint *et al.*, 1979; Schnee *et al.*, 2006; Verheggen *et al.*, 2007; Sasso *et al.*, 2009), it is reasonable to speculate that a reduction of sesquiterpene emissions in cranberries may lead to reduced pest suppression by natural enemies.

In summary, this study provides the first example of potential unintended consequences of plant breeding on direct and indirect defences against insect herbivores. The cross between McFarlin and Potter (Potter has low levels of JA) resulted in an offspring, Stevens, with very low herbivore-induced sesquiterpene emissions; the defence against caterpillars was not affected. Then Stevens was crossed with Ben Lear, which has high levels of inducible sesquiterpene emissions after caterpillar attack but very poor defence. The cross between Stevens and Ben Lear resulted in an offspring, NJS98-23, with higher yields and intermediate plant resistance to caterpillars, but, as its parent Stevens, very low emissions of inducible sesquiterpenes and JA-levels similar to that of Potter. Thus, NJS98-23 may have inherited low sesquiterpene emissions from Stevens, low JA-levels from Potter, and poor caterpillar defence from Ben Lear. While our study used only a single breeding line of cranberries, the results suggest that breeding for traits associated with fruit quality and quantity in cranberries causes distinct, but not entirely predictable, effects on plant defences. Future studies with additional lines are needed to investigate whether the observed reduction in herbivore resistance reflects underlying physiological trade-offs between reproductive- and defence-related traits. Similar research on breeding lineages of other domesticated plants would provide a broader perspective on the effects of breeding on plant defence in agroecosystems and the ramifications for management and the design of future breeding programmes.

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