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Allometric analysis of the induced flavonols on the leaf surface of wild tobacco (*Nicotiana attenuata*)

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

Trichomes excrete secondary metabolites that may alter the chemical composition of the leaf surface, reducing damage caused by herbivores, pathogens and abiotic stresses. We examined the surface exudates produced by *Nicotiana attenuata* Torr. Ex Wats., a plant known to contain and secrete a number of secondary metabolites that are toxic or a deterrent to herbivorous insects. Extractions specific to the leaf surface, the trichomes, and the laminar components demonstrated the localization of particular compounds. Diterpene glycosides occurred exclusively in leaf mesophyll, whereas nicotine was found in both the trichomes and mesophyll. Neither rutin nor nicotine was found on the leaf surface. Quercetin and 7 methylated derivatives were found in the glandular trichomes and appeared to be excreted onto the leaf surface. We examined the elicitation of these flavonols on the leaf surface with a surface-area allometric analysis, which measures changes in metabolites independent of the effects of leaf expansion. The flavonols responded differently to wounding, methyl jasmonate (MeJA), herbivore attack and UV-C radiation, and the response patterns corresponded to their compound-specific allometries. Finding greater amounts of quercetin on younger leaves and reduced amounts after herbivore feeding and MeJA treatment, we hypothesized that quercetin may function as an attractant, helping the insects locate a preferred feeding site. Consistent with this hypothesis, mirids (*Tupiocoris notatus*) were found more often on mature leaves sprayed with quercetin at a concentration typical of young leaves than on unsupplemented mature leaves. The composition of metabolites on the leaf surface of *N. attenuata* changes throughout leaf development and in response to herbivore attack or environmental stress, and these changes are mediated in part by responses of the glandular trichomes.

Keywords: Induced defenses; Quercetin; Quercetin 3-methyl ether; Quercetin 3-methyl ether; Quercetin 3,5-dimethyl ether; Quercetin 3,7-dimethyl ether; Quercetin 3,3′,4′-trimethyl ether

1. Introduction

Secondary metabolites are thought to protect plants growing in nature against herbivores, pathogens or abiotic challenges such as ultraviolet (UV) radiation (Karban and Baldwin, 1997). Predictably, the plant surface, the first point of contact the plant has with environmental hazards, is enriched in secondary metabolites (Juniper and Southwood, 1986). Glandular trichomes, particularly those with photosynthetic capabilities, can devote prodigious amounts of resources to the production of secondary metabolites that influence the chemical composition of the leaf surface (Wagner, 1991). For example, photosynthetic trichomes isolated from tobacco (*Nicotiana tabacum*) have been shown to fix carbon dioxide in order to produce the characteristic diterpenes (Keene and Wagner, 1985). These and other trichome constituents protect the plant against external stresses (Rodriguez and Healey, 1984; Wagner, 1991).

Glandular trichomes are thought to function primarily as a constitutive defense. However, environmental stress as well as the wound hormone jasmonic acid (JA) or its ester, methyl jasmonate (MeJA), can increase the production of metabolites from trichomes (Laue et al., 2000; Tattini et al., 2000). MeJA-elicitation

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of the roots of *N. repanda* caused a rapid increase in the production and release to the surface of *N*-acyl-nornicotines that are about 1000 fold more toxic than nicotine to the specialist herbivore *Manduca sexta* (Laue et al., 2000). The ability to rapidly deploy chemicals to the leaf surface may help a plant to optimize its allocation to defense, particularly if externalizing metabolites reduce or prevent attackers from accessing other tissues. However, not all induced changes in surface chemistry always benefit the plant. For instance, the JA-elicited increase in furanocoumarins on the leaf surface of *Apium graveolens* stimulates the oviposition of the carrot fly (Stanjek et al., 1997), which presumably increases the amount of damage from this herbivore. Hence, understanding how glandular trichomes respond to various external challenges will elucidate how secondary metabolites mediate a plant's interactions with its environment.

The exudates of the glandular trichomes of many *Nicotiana* species contain compounds such as duvane diterpenes and acylsugars, which are known to function defensively against herbivores and pathogens (Bailey et al., 1975; Neal et al., 1994; Severson et al., 1985). In *N. attenuata*, herbivore attack and JA elicitation dramatically changes the amounts of secondary metabolites in whole-leaf extracts (Keinänen et al., 2001). Here we explore which of these induced changes can be attributed to glandular trichomes. We identified a suite of methylated flavonols in the glandular trichomes, which after elicitation by MeJA, herbivore attack, and UV-C exposure, are excreted onto the leaf surface. Flavonols are thought to protect plants against UV-induced damage, pathogen and herbivore attack, as well as oxidative stress, and their accumulation may be elicited by these stresses (Grotewold et al., 1998; Harborne and Williams, 2000; Shirley, 1996). Since compounds from *N. attenuata* are deposited onto a leaf surface that expands during ontogeny, we examine the dynamics of the accumulation of these compounds in a surface-area allometric analysis, a procedure that deserves a brief discussion.

We regress the total quantity (pool) of compounds extracted from the leaf surface against the leaf area from a suite of leaves harvested in different stages of expansion. Such regressions reveal whether a metabolite pool is maintained ($\alpha = 0$), increased ($\alpha = +$), or decreased ($\alpha = -$) as a leaf expands during ontogeny (Fig. 1). Once the allometric pattern of a metabolite is established, the effect of the various elicitors on the slope and Y-intercept of the allometries can be evaluated. For example, an increase in the Y-intercept of a compound with an allometric slope of 0 reflects an increased accumulation of a metabolite early in the ontogeny of a leaf in response to the environmental stress, which remains unchanged during leaf expansion.

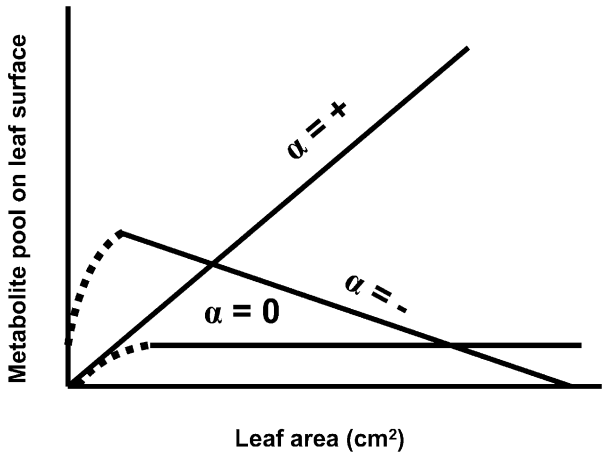


Fig. 1. Categories for leaf surface-area allometries for pools of secondary metabolites on leaves. When $\alpha = 0$, the metabolite pool is synthesized early in leaf development (the initial rapid, short-term increase) and maintained as the leaf expands; for these metabolites, the Y-intercept estimates the pool size. When $\alpha = +$ the leaf continuously produces and increases the surface metabolite pool throughout expansion. When $\alpha = -$, young small leaves sustain a larger metabolite pool, which is subsequently lost as leaves mature. In the latter two allometries, both the slope and Y-intercept may be influenced by environmental factors.

2. Results and discussion

2.1. Compounds extractable from the leaf surface

Sequential and serial extractions (see Experimental) of leaf surface, trichomes and laminar components demonstrated the localization of particular compounds (Fig. 2). With the sequential analysis, the compounds extracted from the surface-only, trichomes-only, or leaf-only demonstrated that each fraction accounted for the expected amount of the total pool of secondary metabolites found when the whole leaf was homogenized. Diterpene glycosides (Fig. 2, No. 3) occurred exclusively in leaf mesophyll (including intact epidermis), whereas nicotine (Fig. 2, No. 1) was found in both trichomes and mesophyll. As expected from polarity, neither rutin nor nicotine (Fig. 2, Nos. 2 and 4) were found on the leaf surface. This pattern of localization was confirmed by the serial washes. As anticipated, the surface washes of the serial and sequential collections were quantitatively identical. When the surface extract was prepared after crushing the trichomes, its composition was similar to the combined sequential collections of surface-only and trichome-only extractions. Acetonitrile (ACN) extraction of an intact leaf (serial collection of the surface, trichome and leaf) had a total pool of secondary metabolites similar to the sum of the individual extractions of surface-only, trichome-only and leaf-only samples. For example, the sum of the quantities of caffeoylputrescine (Fig. 2, No. 2) in surface only (22.1 ± 10.9), trichome only (77.2 ± 3.3) and leaf only (401.6 ± 28.2) extracts was similar to the surface + trichome + leaf

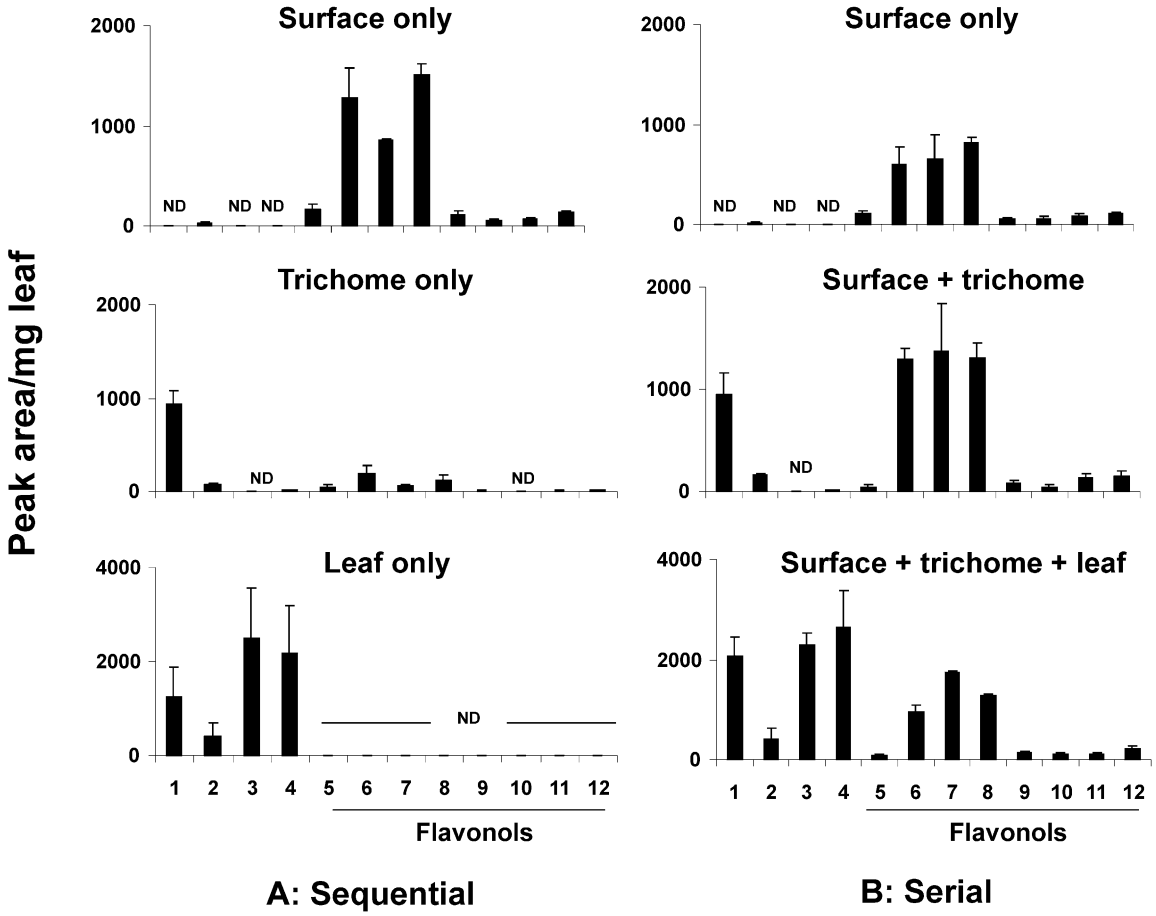


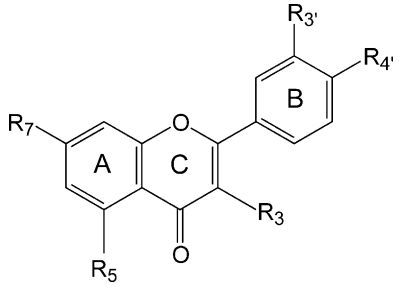
Fig. 2. (A) Sequential analysis of surface, trichomes and internal leaf compounds by sequentially removing components from a single leaf ($n=5$ *Nicotiana attenuata* leaves) and (B) serial analysis of the surface only, the surface + trichomes and the surface + trichomes + internal leaf constituents ($n=5$ *N. attenuata* leaves). Values are mean areas (±S.E.) measured at 254 nm of: (1) nicotine (10×), (2) caffeoylputrescine, (3) diterpene glycosides (10×), (4) rutin (10×), (5) quercetin, (6) quercetin-3-methyl ether, (7) quercetin 4′-methyl ether, (8–10) quercetin dimethyl ether, quercetin trimethyl ether (ND = not detected). Areas rather than absolute amounts were used because of the differences in absorbance maxima of the compounds.

extraction (513.8±20.4). The additivity of the sequential extractions demonstrated that the ACN extraction removed only surface constituents.

To determine if the water content of the leaf lamina influenced the composition of metabolites extracted by ACN, leaf surface and trichomes were prepared with 60% ACN in water as the solvent. The composition of these extracts was identical to those prepared with 100% ACN (data not shown).

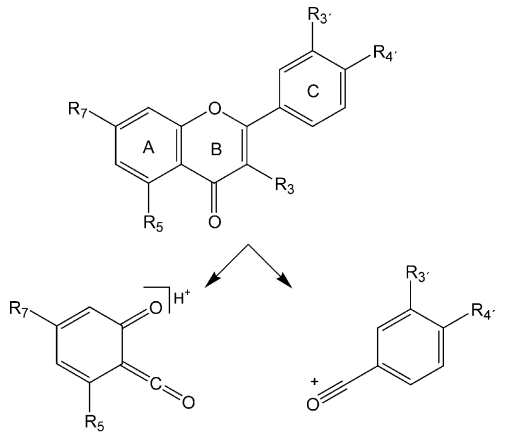
The LC–MS analysis identified eight surface-specific flavonols: quercetin, two monomethylated ethers, three dimethylated ethers and two trimethylated ethers of quercetin (Table 1). The identity of quercetin and quercetin 4′-methyl ether was established by comparing spectra and retention times to commercially available standards. The methyl positions of the other compounds were deduced from their fragmentation patterns (Table 2, Experimental). Quercetin 3-methyl ether was infered because neither RDI nor RDII contained a methyl group, therefore the methyl group was located in the R3 position. The fragmentation patterns of the

Table 1
Quercetin skeleton showing the location of methyl groups (Me) of 8 quercetin-based compounds isolated from *Nicotiana attenuata*



Compound	R_3	R_5	R_7	$R_{3'}$	$R_{4'}$
Quercetin	OH	OH	OH	OH	OH
Quercetin 3-methyl ether	OMe	OH	OH	OH	OH
Quercetin 4′-methyl ether	OH	OH	OH	OH	OMe
Quercetin dimethyl ether 1	OMe	OH	OH	OMe or OMe	
Quercetin dimethyl ether 2	OMe	OMe or OMe		OH	OH
Quercetin dimethyl ether 3	OMe	OMe or OMe		OH	OH
Quercetin 3,3′,4′-trimethyl ether	OMe	OH	OH	OMe	OMe
Quercetin trimethyl ether 1	OMe	OMe or OMe		OMe or OMe	

Table 2
Fragment ions (m/z) of the quercetin aglycone A and B rings used to identify the location of the methoxy group(s). [MW = molecular weight; RT = retention times (min) from HPLC analysis]



RD I **RD II**

Compound	RT	MW	RD I	RD II
Quercetin	21.1	302	153	137
Quercetin 3-methyl ether	21.4	316	153	137
Quercetin 4′-methyl ether	23.0	316	153	151
Quercetin dimethyl ether 1	23.5	328	153	151
Quercetin dimethyl ether 2	24.9	328	167	137
Quercetin dimethyl ether 3	26.7	328	167	137
Quercetin 3,3′,4′-trimethyl ether	25.5	342	153	165
Quercetin trimethyl ether 1	27.4	342	167	151

three quecetin dimethyl ethers showed that in all cases one of the methyl groups was located on the R3 position. The fragmentation patterns also indicated that for quercetin dimethyl ether 1 (RT = 23.5), the second methyl group occurred on either the R3′ or R4′ position. Quercetin dimethyl ether 2 (RT = 24.9) and quercetin dimethyl ether 3 (RT = 26.7) both contained a methyl group on RDI indicating that these compounds were quercetin 3,5 dimethyl ether and quercetin 3,7 dimethyl ether. Similarly, the identity of quercetin 3,3′4′-trimethyl ether was established by finding two methyl groups on RDII and none on RDI while the fragmentation pattern of quercetin trimethyl ether 1 contained a methyl group in the R3 position, one on RDI and one on RDII. Further work is needed to determine which flavonol corresponds to each peak. Analysis of individual trichome collections confirmed that these flavonols occurred in trichomes (data not shown). Previous studies have shown that trichomes excrete flavonoids and that quercetin-3-methyl ether, quercetin 3,7-dimethyl ether and quercetin 3,3′- dimethyl ether occur in *Nicotiana* exudates (Yang et al., 1960; Wollenweber and Dörr, 1995).

Flavonoids located on the leaf surface are very often *O*-methylated and lipophilic (Harborne and Williams, 2000). Such hydrophobic aglycones can be toxic to the plant if retained in the cytoplasm due to their high reactivity (Rice-Evans et al., 1997). Therefore, these constituents are often sequestered in vacuoles (Grotewold et al., 1998; Hrazdina, 1992; Klein et al., 1996; Li et al., 1997). In addition, glycosylation of flavonols may render the molecule less reactive towards free radicals in addition to being more water-soluble, which also facilitates storage (Rice-Evans et al., 1997). In *N. attenuata,* 6 of the 7 quercetin-based methyl ethers extracted from the leaf surface had a methoxy subsitution at R3, the same position as the diglycoside substitution of rutin. Therefore, the majority of quercetin produced by a leaf is conjugated at R3, either with rutinose as rutin and stored in the mesophyll or with a methyl group and externalized, possibly as a means of decreasing the reactivity of the aglycone.

After determining that the ACN leaf wash reflected only flavonols externalized on the surface, we examined the surface area allometries of these constituents. The quercetin methyl ethers displayed all 3 possible allometric patterns (Figs. 3A–C). Leaf surface extracts from younger leaves had more quercetin than older leaves, a negative allometric slope, indicating a possible dilution by growth without sufficient new synthesis or its conversion to a different metabolite (Fig. 3A). Dimethyl ether 1 and the two trimethyl ethers exhibited positive relationships between the amounts of compound produced and leaf area (Fig. 3B), suggesting that synthesis

was higher than leaf expansion. Interestingly, these quercetin derivatives all contained multiple methoxy groups with at least one located on the B ring. Quercetin 3-methyl ether, quercetin 4′-methyl ether, quercetin 3,5-dimethyl ether, and quercetin 3,7-dimethyl ether showed a slope of $\alpha = 0$, suggesting that at an early stage during ontogeny the trichomes cease further production of these metabolites or that their synthesis equaled their loss from the leaf surface (Fig. 3C).

The different allometric patterns of quercetin methylation may be due to the activity of specific methyl transferases. For example, flavonol O-methyltransferase isolated from *Chrysosplenium americanum* exhibited strict specificity for the 3′ position of 3,7,4′-trimethyl quercetin and did not accept the mono- or dimethyl analogs or the parent aglycone quercetin (Seguin et al., 1998). The enzyme is likely involved in the later steps of polymethylated flavonol synthesis in this plant.

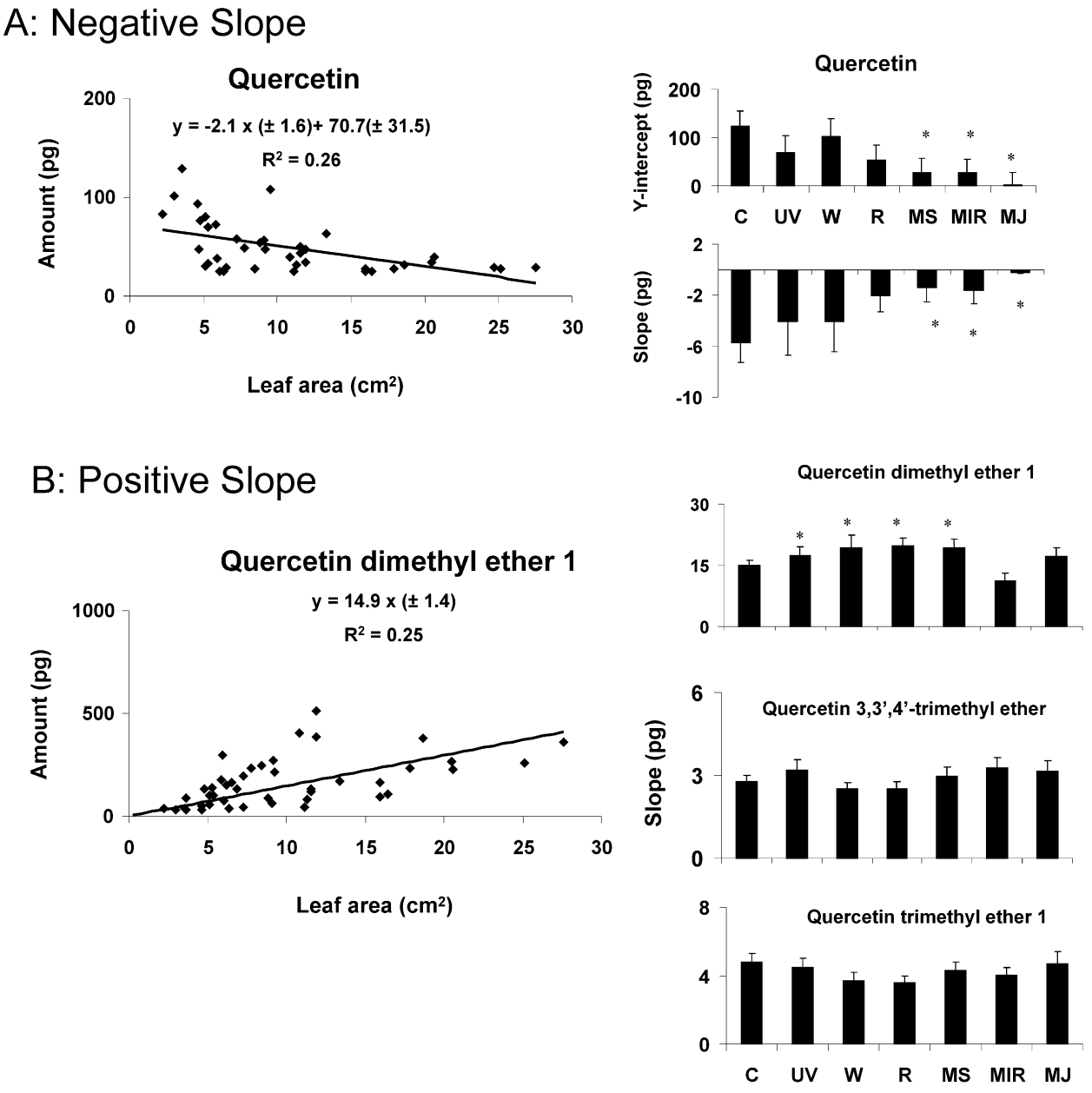


Fig. 3. (A) Negative ($\alpha = -$), (B) positive ($\alpha = +$), and (C) zero ($\alpha = 0$) slope (pg/cm^2) allometries of quercetin and 7 of its methyl ethers extracted from the leaf surface of *N. attenuata*. Plants were not treated (C), irradiated with UV-C radiation (UV), wounded (W), wounded + *M. sexta* regurgitant (R), or fed on by *M. sexta* larvae (MS) or by mirids (MIR), or treated with 250 μg methyl jasmonate (MJ). Surface-area allometric relationships are presented for non-treated plants. Effects of treatments were determined by comparing the slopes of the flavonols with either significantly positive or negative slope (A and B), or Y-intercept for flavonols with a zero slope (C). (Bars represent mean ± S.E. in pg based on calculated pmol values from a quercetin standard curve corrected for the molecular weight contributions of the methylation; * indicates significant difference from untreated controls at $P < 0.01$ as determined by ANCOVA.)

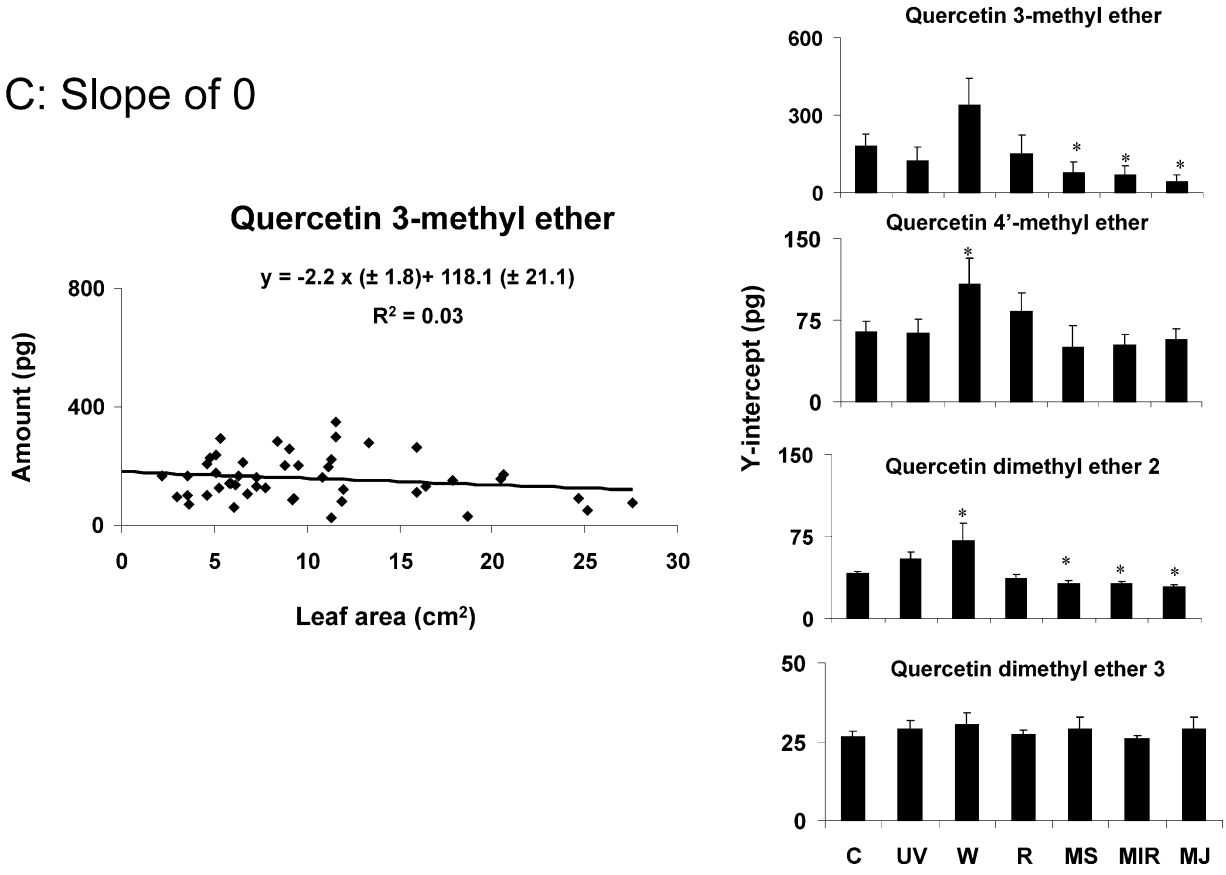


Fig. 3 (*continued*).

2.2. Flavonol response to environmental stress

Quercetin and the 7 methylated derivatives responded differently to wounding, MeJA, herbivore attack, and UV radiation, and patterns arose that corresponded to their allometries. Compared with control plants, wounding increased the amount of quercetin 3-methyl ether, quercetin 4′-methyl ether and quercetin dimethyl ether 2 which were compounds that had an $\alpha = 0$ (Fig. 3B). Interestingly, plants treated with MeJA, which elicits responses similar to wounding and herbivore attack in *N. attenuata* (see Ref. in Baldwin, 2001), had lower amounts of quercetin 3-methyl ether and quercetin dimethyl ether 2 as well as quercetin (Fig. 3A and B). *Manduca sexta* and mirid feeding also decreased the amount of these 3 compounds (Fig. 3A and B). Previous work with tomato (*Lycopersicon esculentum*) has shown that jasmonate-treatment reduced the content of tannins (Bialczyk et al., 1998) and that the production of catechin, (−) epicatechin, quercetin, and quercetrin was not enhanced by either radiation or MeJA treatments (Rudell et al., 2002). For the flavonols showing a positive slope (Fig. 3B), UV-C, wounding, wounding plus regurgitant and *M. sexta* feeding all increased the amount of dimethyl ether 1 produced.

The diversity of responses shown by quercetin and its methyl ethers is not surprising given the many roles that flavonols are likely to play in plant defense (Harborne and Williams, 2000). Localizing flavonols to the leaf surface in trichomes could provide protection against damaging UV-B radiation, and *O*-methylation may provide additional benefit by shifting the UV absorption properties to shorter wavelengths (Harborne and Williams, 2000). For example, *Gnaphalium vira-vira* increased the synthesis of the 7-*O*-methylaraneol at the expense of araneol (5,7-dihydroxy-3,6,8-trimethoxyflavone) and increased the amount of 7-methyl ether present in the surface extract after twenty days of UV-B radiation (Cuadra and Harborne, 1996).

Protection against microbial attack is a well-established function of flavonoids and related polyphenols. They can act as constitutive agents or they may accumulate in response to microbial invasion (Grayer and Harborne, 1994; Harborne, 1999a). Quercetin, quercetin 3,7-dimethyl, and 3,3′-dimethyl ethers have antibacterial and antiviral activity (Malhotra et al., 1996). Recently, the deglucosidation of rutin and subsequent formation of quercetin has been implicated in the browning of tobacco leaves and onion scales and may be related to the formation of hydrogen peroxide and anti-microbial compounds (Takahama et al., 2001).

Flavonoids play a role in protecting plants from herbivory (Beninger and Abou-Zaid, 1997; Oberdorster et al., 2001); however, they may also function as ovipostion and feeding stimulants (Fulcher et al., 1998). For example, rutin acts as a phagostimulant for the locust, *Schistocerca americana* (Bernays et al., 1991) but deters gypsy moths (*Lymantria dispar*) and European corn borers (*Ostrina nubilalis*) (Abou-Zaid et al., 1993). Rutin stimulates feeding of *M. sexta* more than a mixture of quercetin and the sugar rutinose (de Boer and Hanson, 1987). Quercetin 7-methyl ether isolated from *Flourensia thruifera* did not deter the feeding behavior of *Spodoptera littoralis* (Faini et al., 1997). Interestingly, quercetin 7-methyl ether decreased the growth of European corn borer larvae (Harborne, 1999b). Clearly, the role that flavonols play in defending the plant against insect attack is complex and likely species-specific.

2.3. Insect behavior bioassay

In our study, insect herbivory decreased the amount of quercetin and the quercetin mono-methyl ethers, suggesting that the plant may reduce the production of these compounds in order to decrease a potential phagostimlatory response. Mirids are one of the most prevalent herbivores of *N. attenuata* in its natural habitats (Kessler and Baldwin, 2001) and their feeding tends to be concentrated on the younger leaves located in the center of a rosette plant where we have observed them feeding on trichomes (A.L. Roda, personal observation). Because quercetin was found in higher amounts on these leaves, we hypothesized that the quercetin may function as an attractant that could help the insects locate a preferred feeding site. Consistent with this hypothesis, mirids were found more often on mature leaves sprayed with quercetin to a concentration typical of young leaves than on mature leaves sprayed with water containing only the carrier (Fig. 4). Mirids, however, showed no preference for mature leaves sprayed with quercetin 4′-methyl ether (Fig. 4). These results suggest that mirids may use quercetin to locate younger and potentially more nutritious food source (Kessler and Baldwin, in press).

Mirids tended to reduce all the flavonols examined in this study (Fig. 3). The continuous feeding of mirids on the trichomes may have simply reduced the amount of these compounds found on the leaves and had little to do with the plant altering flavonol synthesis or methylation. This hypothesis does not exclude a role these flavonols may have in insect-plant interactions. *Manduca sexta* feeding, as well as MeJA elicitation, reduced the accumulation of quercetin and mono-methylated ethers. Because only non-treated leaves were included in the analysis, these observed responses were systemic and were not due to the loss of trichomes or trichome exudates. In addition, caterpillar feeding increased the production of

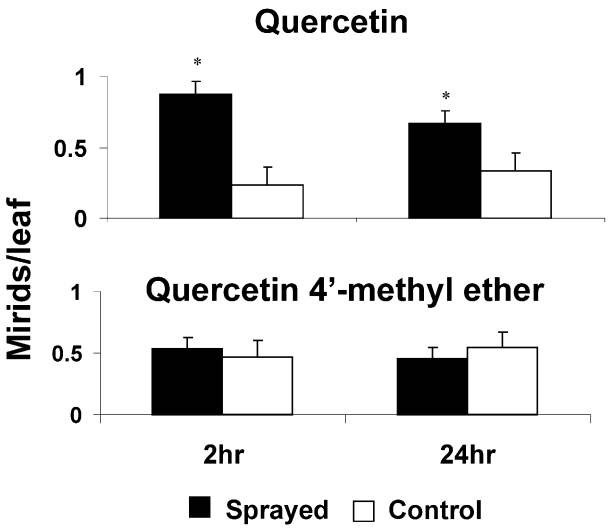


Fig. 4. Mean (±S.E.) of mirids recorded on mature, fully expanded leaves sprayed with 90 ng quercetin or 180 ng quercetin 4′-methyl ether (quantities typical of young leaves preferred by mirids) and on control leaves sprayed only with the flavonol carrier 2 and 24 h after time 0 when the mirid was placed on the leaves ($n = 20$ for each paired comparison; * = $P < 0.01$ as determined by t-test).

dimethyl ether 1, which implies a possible function in plant-herbivore interactions. Clearly, further studies are needed to discern how these compounds influence herbivores and whether alterations in their production benefit the plant.

2.4. Conclusion

Glandular trichomes are responsive to environmental stresses and these responses can change the metabolite composition of the leaf surface throughout development. The differences in allometric responses amongst the differently methylated quercetin derivatives suggest different functions in the plant. Their alterations in accumulation as a result of wounding, insect herbivory, and UV radiation suggest a role in defense. The value of elicitation in understanding secondary metabolite function is only one of Meinhart H. Zenk many enduring scientific legacies.

3. Experimental

3.1. Plant material

Nicotiana attenuata Torr. ex Wats. plants from an inbred line originating from a natural population from Utah (DI ranch, T40S R19W, section 10 1988) were grown for three weeks as previously described (Baldwin and Ohnmeiss, 1994). Rosette plants of similar age, size and number of mature leaves were selected for the experiments and randomly assigned to the treatment

and control groups of 7 plants each. For each plant, we determined the source/sink transition leaf and designated this position as node '0'. All leaves older were given positive numbers reflecting increasing age or negative numbers signifying progressively younger leaves.

3.2. Extraction and compound characterization of metabolites

To determine the location of constituents, the leaf surface, trichomes and the entire leaf were extracted sequentially. To extract the leaf surface, leaves at node 0 were cut at the base of the petiole and rinsed 10 × in 4 ml of ACN on each side of the leaf. After extracting the leaf surface, the trichomes were crushed by squeezing the leaf on the side of a glass beaker and re-extracting the surface as previously described. Finally, the leaf was homogenized in ACN (1 ml). From a second set of replicate leaves, serial extractions were performed: leaves were washed only, washed after breaking the trichomes or homogenized without manipulation. In contrast to these serial extractions, the number and amount of metabolites from the sequential extractions should increase as leaves are progressively disrupted. An internal standard (1.5 μg of 2-chlorobenzoic acid) was added to each vial before samples were concentrated. Extracts were first concentrated under a stream of N_2 to 2 ml, transferred to Eppendorf tubes, and centrifuged for 10 min (13.2×10^3 rpm). The samples were transferred to a second tube, concentrated to 200 μl using Eppendorf concentrator (5301) at 60 °C, centrifuged again for 10 min (13.2×10^3 rpm) and transferred to HPLC vials. Metabolites were separated by HPLC (Hewlett-Packard, Avondale, PA, USA, HP 1100), on a Inertsil (GL Sciences, Tokyo, Japan) ODS-3 column with solvents (A) 0.25% H_3PO_4 in water and (B) ACN eluted with a gradient program of 0% B for 6 min; 12% B 10 min; 18% B for 30 min, 58% B for 40 min; 98% B for 45 min with a flow rate of 1 ml/min, an injection volume of 15 μl, and a column temperature maintained at 24 °C. The eluent was monitored with a DAD detector at 210, 230, 254, 320 and 365 nm. The retention times and UV–vis spectra of nicotine, diterpene glycosides, rutin, and caffeoylputrescine were previously determined (Keinänen et al., 2001).

Surface constituents having a UV spectrum similar to rutin were investigated further with LC–MS using a Hewlett-Packard (Avondale, PA, USA) HP 1100 HPLC coupled to a Micromass Quattro II tandem quadrupole mass spectrometer (geometry quadrupole-hexapole-quadrupole) equipped with an electrospray (ESI) source. The capillary and cone voltages in ESI+ mode were 3.4 kV and 16 V respectively, whereas in ESI- mode the capillary and cone voltages were 3.8 kV and 3.5 V. Nitrogen was used for nebulization (20 l h^{-1}) and drying gas (150 l h^{-1}, 250 °C). Standard and product ion (MS/MS) mass spectra were measured as previously described (Keinänen et al., 2001). Argon was used for collision-induced dissociations at 2×10^{-3} mbar and a collision energy of 28 eV was used for fragmentation. Fragmentation patterns identified these compounds as quercetin and mono-, di- and tri- methylated ethers (Table 2). Positions of the methyl groups were determined using the observed fragmentation patterns of the available standard compounds (quercetin quercetin 7-methyl ether, quercetin 3′-methyl ether and quercetin 4′-methyl ether). In the positive ESI mass spectra of the standards we can observe diagnostic ions from retro Diels-Alder reactions I and II which were found to provide sufficient information for determination of sites of methylation on the rings A and C (Table 2). The pseudo-molecular ions provided information about the number of methyl groups. In the cases where the methyl group cannot be seen in RD I and RD II fragments it was assigned to be at the position 3′ on the B ring. The position of methyl group in monomethylated ring A and C was tentatively assigned based on the observation of intensities of pair of RD I and RD II ions. Quercetin and quercetin 4′-methyl ether (a single methylated ether) were confirmed by comparisons with authentic reference compounds and their HPLC retention times. The amounts of quercetin and its methylated ethers were expressed as peak areas at 360 nm/cm^2 leaf. Because the UV spectra of the methylated derivatives of quercetin were similar to that of quercetin, we converted the pmol values based on a quercetin standard curve to mass values by substituting the molecular weight of quercetin for the molecular weight corresponding to the compounds methylation. The amounts of the other compounds were expressed as peak areas at 210 nm/g fresh leaf mass.

3.3. Insect rearing

Tobacco hornworm (*Manduca sexta,* Lepidoptera, Sphingidae) eggs purchased from Carolina Biological Company (USA) were cultured in climate chambers (27 °C; ca. 60% RH 16:8, light:dark regime) until eclosion. Larvae used for regurgitant collection were reared on *N. attenuata* leaves until 4th instar. Mirids (*Tupiocoris notatus,* Hemiptera, Miridae) collected from an indigenous population in Utah (Kessler and Baldwin, 2001) were reared on *N. attenuata* plants maintained under greenhouse conditions (27 °C; ca. 60% RH 16:8, light:dark regime).

3.4. Flavonol induction in glandular trichomes

Four treatments (wounding; *M. sexta* regurgitant, *M. sexta* larval feeding, MeJA) were applied to node +2 leaves. To avoid breaking trichomes, leaves were wounded

by making 20 punctures on each side of the mid-rib with a metal probe. After wounding, the leaf surface was sprayed with dionized water. *Manduca sexta* regurgitant was sprayed onto wounded leaves (ca. 10 μl of undiluted regurgitant/leaf) as previously described (McCloud and Baldwin, 1997). One 1st instar larvae was placed onto each *M. sexta* treated plant. MeJA (250 μg/plant) was applied in a lanolin paste to the petiole. Each mirid-treated plant received 3 adult females. UV-treated plants were exposed to UV-C radiation (Mineralight® Model UVG-54, UV 254 nm, 115 V, 60 Hz, 0.16 AMPS, UVP, Inc. San Gabriel, CA) for 5 min held at a distance of 50 cm. Plants showed no sign of UV-C exposure (bronzing, etc.) one week following exposure. After treatment, all plants, including non-treated controls were placed in individual glass cages (25 × 25 × 60 cm). Each treatment was replicated 5 ×. After 5 days, +3 through-1 leaves were removed, weighed, washed, and digitized as previously described. Leaf area measurements were made using SigmaScan Pro computer software (SigmaScan Pro, Version 5, SPSS, Inc. Chicago, IL). The amount of each flavonol was regressed against leaf area and analysis of covariance (ANCOVA) was used to compare slopes or intercepts of control plants against each treatment (StatView, 1998).

3.5. Insect behavior bioassay

Two fully expanded nodes +3 and +4 leaves (previously determined to have very low quercetin) were cut at the base of the petiole and wrapped in water-saturated cotton. The leaves were supported in 2.0 ml Eppendorf centrifuge tubes and each pair was placed into an individual glass cage so that their edges overlapped. One leaf of the pair was sprayed with either quercetin (90 ng) or quercetin 4′-methyl ether (180 ng) dissolved in distilled water whereas the other leaf received only the carrier (DMF) dissolved in distilled water (replicated 20 times for each flavonol). After drying, a mirid nymph (3rd or 4th instar) was placed on the leaf pair. Observations of the mirid's location were made 2 and 24 h after placement. The number of mirids found on the treated leaf was compared with the number found on the control for each time period (*t*-test, StatView, 1998).

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