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Evolutionary change from induced to constitutive expression of an indirect plant resistance

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Induced plant resistance traits are expressed in response to attack and occur throughout the plant kingdom[1,2]. Despite their general occurrence, the evolution of such resistances has rarely been investigated[3]. Here we report that extrafloral nectar, a usually inducible trait, is constitutively secreted by Central American *Acacia* species that are obligately inhabited by ants. Extrafloral nectar is secreted as an indirect resistance[4], attracting ants that defend plants against herbivores[5]. Leaf damage induces extrafloral nectar secretion in several plant species[6–8]; among these are various *Acacia* species and other Fabaceae investigated here. In contrast, *Acacia* species obligately inhabited by symbiotic ants[9] nourish these ants by secreting extrafloral nectar constitutively at high rates that are not affected by leaf damage. The phylogeny of the genus *Acacia* and closely related genera indicate that the inducibility of extrafloral nectar is the plesiomorphic or 'original' state, whereas the constitutive extrafloral nectar flow is derived within *Acacia*. A constitutive resistance trait has evolved from an inducible one, obviously in response to particular functional demands.

Induced resistances to herbivores have been described for more than 100 plant species[2] and can greatly benefit plants[7,10–13]. They are generally regulated by the octadecanoid pathway, in which the plant hormone jasmonic acid forms a central signal[2,14,15]. This pathway is taxonomically widely distributed and thus has to be regarded as evolutionarily highly conserved. Therefore, the question arises as to whether the expression regime of induced resistance traits can be evolutionarily adapted to particular functional demands.

We used extrafloral nectar (EFN) secretion by Central American *Acacia* (Fabaceae subfamily Mimosoideae) species to investigate whether a resistance trait can be differently expressed in closely related species. EFN is secreted by all species through glands on the leaf stalks, but it serves two different functions. The obligate myrmecophytes among these species permanently house ant colonies in their hollow thorns[9]. These symbiotic ants defend their host against herbivores and competing vegetation. They are nourished by plant-derived cellular protein-rich food bodies[16] and by EFN, and both the ants and the plants seem to be highly adapted to this mutualism[9,17]. Other, non-myrmecophytic species secrete EFN that is consumed by non-specialized ants from the vicinity. Agrawal & Rutter[18] stated that ants obligately inhabiting myrmecophytes, in general recruit actively to parts of their host that currently require defence. In contrast, attraction of unspecialized ants to non-myrmecophytes was predicted to be controlled by the plants, for example, by short-term increases in the provisioning of food rewards. EFN secreted by *Acacia* functions as a regular food source for specialized mutualists of myrmecophytes and as a 'bait' attracting ants facultatively to non-myrmecophytes, thus allowing a test of these predictions.

We studied five myrmecophytic and four non-myrmecophytic *Acacia* species (all of the subgenus *Acacia*) as well as three species of related genera of the Mimosoideae. Study sites were located at the Pacific coast and in the Isthmus of Tehuantepec (state of Oaxaca, Mexico). Only non-myrmecophytes that grew at the same sites as myrmecophytes were selected, so that putative site effects on EFN secretion patterns could not cause differences among species (see Methods for a description of how EFN flow was induced and quantified). EFN secretion by all non-myrmecophytes (*Acacia cochliacantha*, *A. farnesiana*, *A. macracantha*, *A. pennatula*, *Leucaena leucocephala*, *Piptadenia flava* and *Prosopis juliflora*) increased after mechanically damaging leaves or after jasmonic acid application, and was five to more than ten times higher on treated than on control twigs (Fig. 1). In contrast, EFN secretion did not respond to leaf damage in any of the myrmecophytes (*Acacia chiapensis*, *A. collinsii*, *A. cornigera*, *A. globulifera* and *A. hindsii*, Fig. 1); all species secreted EFN constitutively at high rates. Therefore, the same trait was inducible in some, but constitutive in other species of the same genus, and there was an obvious relation to its different functions that matches the predictions of Agrawal & Rutter[18]. The induction of EFN secretion in non-myrmecophytes can guide non-specialized ants to plant parts that are currently under attack, whereas ants inhabiting myrmecophytes are provided with a permanent EFN flow at rates which in most cases are higher than those in induced non-myrmecophytes (Fig. 1).

EFN secreted by only one myrmecophyte (*A. collinsii*) responded to exogenous jasmonic acid application (Fig. 1), thus raising the question of whether the octadecanoid pathway is active in the species investigated here. Leaves of four myrmecophytes and four non-myrmecophytes were damaged mechanically in the field to

quantify endogenous jasmonic acid levels. Mechanical damage led to a significant increase (repeated measures analysis of variance (ANOVA): $P < 0.05$ for all eight species) in endogenous jasmonic acid in all species investigated (Fig. 2), demonstrating—along with the results of the inhibitor experiment (Fig. 3)—a functioning octadecanoid signalling cascade in all species. Retaining this pathway intact allows the plants to keep all other jasmonic-acid-responsive traits inducible (see refs 14, 15 for the high number of traits regulated through the octadecanoid pathway).

For further investigations of the role of jasmonic acid we used phenidone (1-phenyl-3-pyrazolidinone; Sigma–Aldrich), an inhibitor of lipoxygenases[19]. Endogenous jasmonic acid synthesis by *Acacia* leaves in response to mechanical damage is suppressed to a large degree (but not completely) when leaves are treated 30 min in advance and immediately after mechanically damaging them with a 2 mmol aqueous solution of phenidone (data not shown). The same treatment led to a suppression of the response in EFN secretion to damage in the non-myrmecophytes and to a strong decrease in EFN secretion by myrmecophytes (Fig. 3). EFN secretion (or the response in EFN secretion to damage, respectively) could be restored by exogenous jasmonic acid application, showing that the suppression of EFN secretion by phenidone was indeed due to a suppressed endogenous jasmonic acid synthesis rather than caused by any side effects of phenidone.

Myrmecophytic *Acacia* species exhibit a constitutive EFN secretion; however, the octadecanoid pathway is active in these species. An increase in jasmonic acid, irrespectively of whether endogenous or exogenously applied, did not cause any increase in EFN secretion above the constitutive level in four of the myrmecophytes, even though EFN flow in myrmecophytes depends on jasmonic acid. It is obviously the responsiveness of EFN secretion to jasmonic acid, rather than an earlier part of the signalling cascade, which has been changed in these species. In myrmecophytes the basal levels of jasmonic acid (Fig. 2, levels at $t = 0$ min) are already sufficient to elicit maximum EFN secretion rates; however, lowering the jasmonic acid concentration still causes a decrease in EFN secretion.

How often has the regulation regime of EFN secretion changed during evolution, and which type is the ancestral one; an inducible or constitutive flow of EFN? So far only one study has attempted to reconstruct the phylogeny of an induced resistance. Thaler & Karban[3] reported a repeated evolution of induced resistance to mites in cotton species. Unfortunately, this study suffered from the methodological difficulty of defining resistance as the number of mites developing on a given plant; this led to different results when the threshold separating 'resistant' from 'not resistant' was chosen differently.

The *Acacia* species investigated here belong to the monophyletic
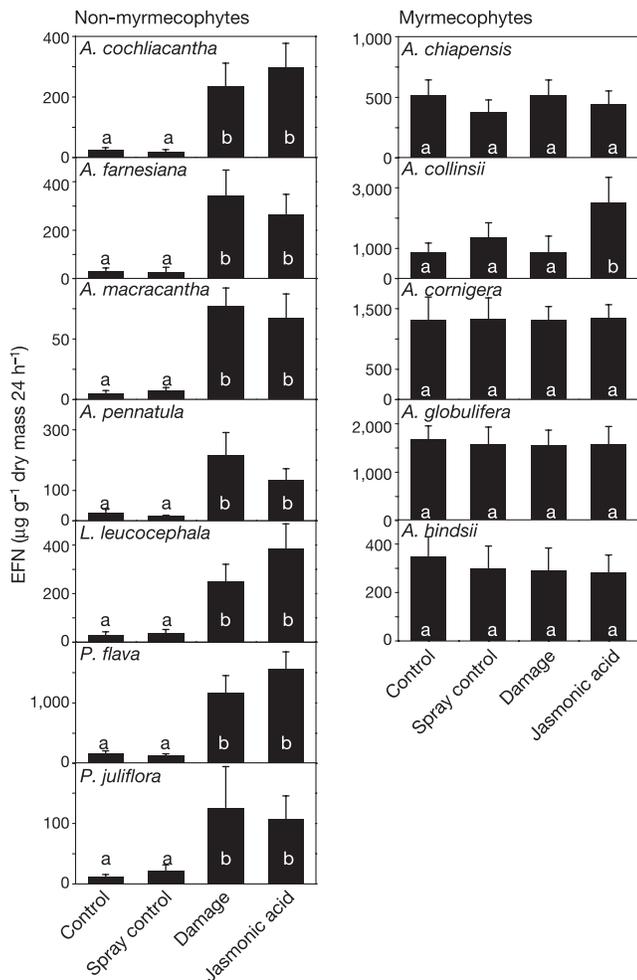
Figure 1 Extrafloral nectar secretion in response to different treatments. EFN secretion rates are given in μg soluble solids per g leaf dry mass per 24 h. Treatment (control, untreated control; spray control, water sprayed on leaves; damage, leaves mechanically damaged; jasmonic acid, aqueous solution of jasmonic acid sprayed on leaves) had a highly significant effect on EFN secretion by all non-myrmecophytes and *A. collinsii* (repeated measures ANOVA: $P < 0.01$). Different letters indicate significant differences among treatments ($P < 0.05$ according to least significant difference (LSD) post-hoc analysis). Sample size = 10 individual plants per species. Error bars indicate standard errors.
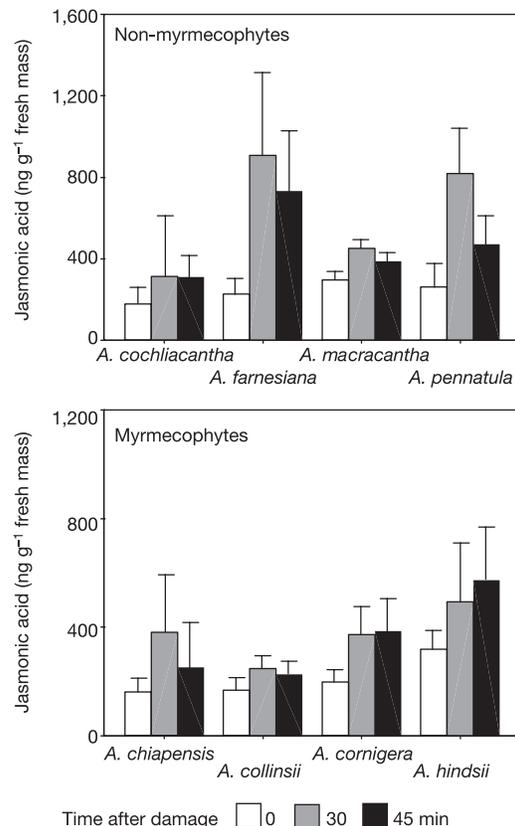

Figure 2 Endogenous concentrations of jasmonic acid. Endogenous jasmonic acid (ng per g fresh mass) in leaves of eight *Acacia* species at different times after mechanically damaging leaves. Sample size = 3 plant individuals per species. Error bars indicate standard errors.

subgenus *Acacia*, in which the neotropical representatives are embedded as a distinct clade[20,21] (see Supplementary Materials). Inducible EFN secretion was observed in several species of subgenus *Acacia* as well as in non-*Acacia* species and has recently also been described for *Phaseolus lunatus*, a leguminous species belonging to the subfamily Faboideae[6]. Constitutive EFN flow was observed only in myrmecophytic *Acacia* species, indicating that this is the derived state. However, the myrmecophytic Acacias investigated are not included in published molecular phylogenetic surveys. To confirm their phylogenetic position, comparative sequencing of the *trnL*–*trnF* region—including the *trnL* intron and the spacer between the *trnL* and *trnF* genes—and of the *trnK* intron of the chloroplast DNA, was conducted for up to three individuals for the species studied (except *A. globulifera*) (see Methods). The alignment, using sequences for both loci of *Mimosa tenuiflora* (gi32365198 and gi27923094, ref. 21) as the outgroup, resulted in 3,302 aligned positions. Two hundred positions contained gaps indicating 28 insertions or deletions ('indels'). By eliminating length mutations in the polyA and polyT regions, five indels were potentially informative, only one of which is located within the species of *Acacia* studied. Employing indels as characters in phylogenetic analyses did not alter the tree topology. Maximum parsimony analysis yielded 57 most parsimonious trees with a length of 269 steps, a consistency index (CI) of 0.88 and a retention index (RI) of 0.83. The myrmecophytic *Acacia* taxa investigated are monophyletic within the subgenus *Acacia*, and, with *Mimosa* as the outgroup, *Piptadenia*, *Prosopis* and *Leucaena* are subsequent sisters to the *Acacia* species analysed (Fig. 4). This relationship is confirmed when sequences are analysed together with published data (Supplementary Figs). *A. farnesiana* (inducible EFN flow) is intercalating between a group containing inducible and constitutive species, indicating a paraphyly of inducible EFN secretion within
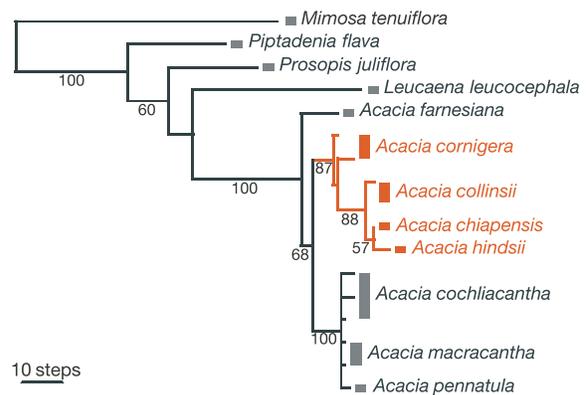
Acacia and thus supporting the assumption that this character is the ancestral state.

Additional support for the relationships, as observed based on chloroplast DNA sequences, was obtained by further analysis of genetic similarities using amplified fragment length polymorphism (AFLP) fingerprinting. AFLP markers are mainly composed of nuclear loci and thus reflect nuclear inheritance. Two hundred and eighteen highly polymorphic band levels were achieved by using four primer pairs. Different individuals of the same species clustered together in all cases, and the results of the cluster analysis corresponded in general to the maximum parsimony analysis of chloroplast loci (Fig. 4). The species exhibiting constitutive EFN flow form one cluster together with some species showing inducible EFN flow, whereas *A. farnesiana*, *P. flava*, *P. juliflora* and *L. leucocephala* (all inducible) form distinct clusters. Myrmecophytic *Acacia* species are positioned in two clusters and in this respect are not in congruence with the chloroplast DNA phylogeny.
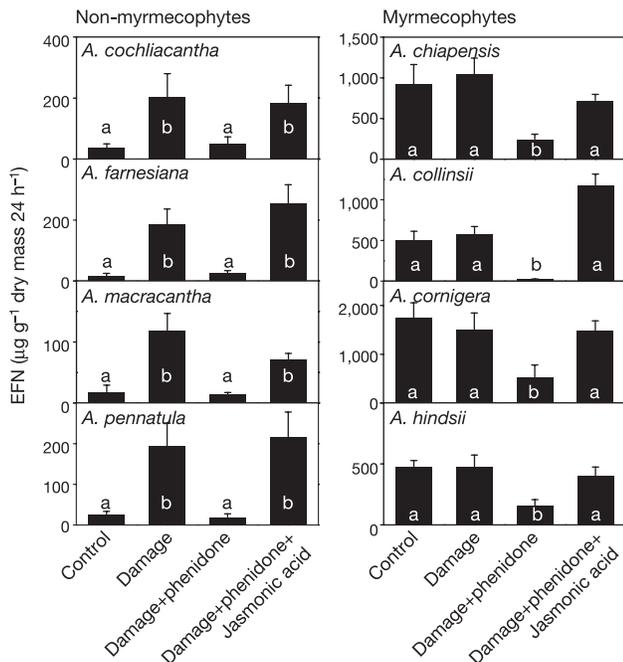

Figure 3 Extrafloral nectar secretion rates after inhibition of jasmonic acid synthesis. Endogenous jasmonic acid synthesis was inhibited by application of phenidone, and EFN secretion (μg soluble solids per g leaf dry mass per 24 h) was quantified one day later. Treatment significantly affected EFN secretion (repeated measures ANOVA: $P < 0.01$ for all species). Different letters indicate significant differences among treatments ($P < 0.05$ according to LSD post-hoc analysis). Sample size = 10 plant individuals per species. Error bars indicate standard errors.
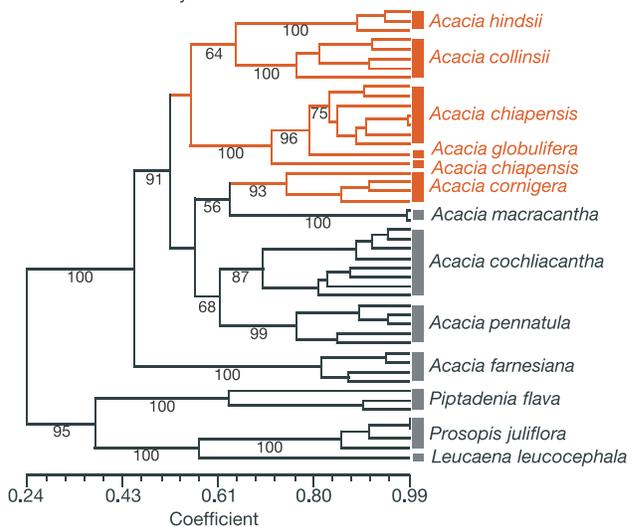
Figure 4 Phylogenetic relationships. **a**, **b**, Phylogenetic reconstruction based on a combined data matrix of the chloroplast DNA markers *trnL*–*trnF* region and *trnK* intron (**a**) (as a strict consensus tree from the 57 most parsimonious trees, shown as a phylogram), and genetic similarities among investigated species as revealed by cluster analysis of AFLP data (**b**) (as UPGMA from the Dice coefficient matrix). Myrmecophytic species (constitutive nectar secretion) are marked red. For both data sets several individuals have been analysed from most species. Equal sequences had been united into one taxonomical unit. Bootstrap values are given below the corresponding branch. Lengths of branches indicate the number of mutation steps (bar: ten steps) in the displayed phylogram.

A. cornigera appeared to be very similar to *A. macracantha* and was therefore nested within the non-myrmecophytic Acacias. Fingerprint analysis can be influenced by introgression of single loci. The high similarity of *A. cornigera* with *A. macracantha* could thus result from hybridization[22]; however, this relationship is only weakly supported by bootstrap analysis.

In the AFLP fingerprints, the dissimilarity among species with induced EFN flow was higher than among species with constitutive EFN flow; however, both groups are comparable in numbers of both species and samples. This difference and the order of the corresponding samples in the cluster analysis confirmed the main result of chloroplast DNA sequencing; that is, the young age and derived character of constitutive EFN secretion.

For cotton, Thaler & Karban[3] reported inducibility of resistance to be the derived state. In *Acacia*, it is obviously the constitutive EFN secretion that emerged as a derived trait during the evolution of myrmecophytism, showing a functional change in the way host plants guide defending animals while a facultative mutualism has been converted to an obligate one. Quantitative variation in the level of induced resistance traits have been reported, for example, for volatiles[23] or secondary metabolites[24]. In contrast, our study shows qualitative differences: the inducible phenotype of a resistance trait has evolved to a constitutive one in response to modified functional demands.   □

Methods

All the plants investigated were shrubs 1–2.5-m-tall, growing under field conditions. Species were determined following Janzen[9] and by comparison with voucher specimens from the Herbarium MEXU, UNAM, Mexico City.

Induction and inhibition of EFN secretion

Ten plants per species were used in each of the two experiments on EFN induction. Four twigs per plant were matched for age, within-plant location and leaf number, and randomly subjected to different treatments. The five youngest fully expanded leaves of each twig were protected from nectar consumers by applying Tangletrap (Tanglefoot) around the twig base and putting the twigs into gauze bags. 'Control' leaves received no additional treatment. 'Damage' leaves were treated with a metal brush resulting in three to five holes per leaflet. On the 'jasmonic acid' leaves, an aqueous 1 mM solution of jasmonic acid was sprayed until there was runoff, and the same amount of water was sprayed on 'spray control' leaves. 'Damage + phenidone' leaves were sprayed two times with a 2 mmol aqueous solution of phenidone 30 min before and immediately after mechanically damaging them as described above, and 'damage + phenidone + jasmonic acid' received two additional sprays of jasmonic acid solution during the 30 min after the final phenidone application. EFN secretion was quantified in all cases 24 h after treatment as described previously[7,25]. For quantification of endogenous jasmonic acid levels, leaves were damaged as in the 'damage' treatment and were harvested at $t = 0$ min and after 30 and 45 min. Extraction and quantification of endogenous jasmonic acid followed the protocol of Koch *et al.*[26]. Shortly afterwards, 1.0 g of leaf tissue was frozen and $[9,10-^2H_2]$ dihydro-jasmonic acid was added as an internal standard[27]. Jasmonic acid was extracted and purified using NH_2-propyl SPE cartridges (Varian). Detection and quantification of jasmonic acid was done by GC-SIM-MS (gas chromatography-selected ion monitoring-mass spectrometry) without further purification.

Phylogenetic analysis and genetic distances

Leaves were collected in the field and dried over silica gel at ambient temperature. DNA was extracted from roughly 40 mg of dry leaf material with DNeasy Plant Mini Kit (Qiagen). AFLP analysis was performed according to the protocol provided with the 'AFLP Core Reagent Kit' (Invitrogen) using the restriction enzymes *Eco*RI and *Mse*I. Four primer pairs differing by their selective nucleotides (E_{AAC} versus M_{CTC}, M_{CAT} and M_{CTA}, and E_{AAG} versus M_{CAT}) were used for the final polymerase chain reaction (PCR) amplification. The PCR products were analysed in 5% Urea-PAGE. AFLP patterns were revealed by exposure to X-ray films (LifeRay, Ferrania). Band levels were scored as present (1) or absent (0). Estimation of genetic similarities and cluster analysis were performed with the NTSys pc Version 2.10 (Applied Biostatistics) using the Dice coefficient (corresponding to Nei-Li distances) for the analysis of banding patterns (Manual for NTSys pc). Cluster analysis was performed as unweighted pair group method using arithmetic averages (UPGMA).

For analysing chloroplast DNA sequences, amplification was performed as described previously[28]. The entire *trnL–trnF* region was amplified using the primers C and F (ref. 29), and the *trnK* intron was amplified in two parts using the primers 2-trnK-3914F and 16-trnK-2R (ref. 30) and the internal primers AC1100F (GCCGTCAGTGTGGAAATTCC) and AC1300R (CAGTATCGAAGGGTTTGAATC), designed after comparison with published sequences for *Acacia*[21]. Sequencing was performed using an ABI 377 (Applied Biosystems) according to the manufacturer's protocol, applying the same primers as for amplification. For the *trnK* intron AC1700F (GGAAAATCCATTCTGGCTTC) was used as an additional sequencing primer. The sequences were aligned manually and maximum parsimony analysis was performed using PAUP 4.0b10 (ref. 28) for the single data sets and

a combined matrix from both loci. In addition, maximum likelihood analysis and neighbour joining with PAUP were performed and revealed analogous results.

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