

and maintenance of high diversity (1–4, 20, 31, 32), even though the specific mechanisms by which edaphic factors might influence diversity have, until now, never been tested (33). Our results suggest a mechanism by which herbivores may influence plant evolution: by accentuating habitat differences and thereby increasing the potential for edaphic heterogeneity to produce habitat specialization. There is mounting evidence that parapatric speciation across environmental gradients can occur in the face of gene flow (34–36). Thus, our data suggest that herbivores can be viewed as a diversifying force because they make existing abiotic gradients more divergent and cause finer-scale habitat specialization by magnifying the differences between habitats. Our study highlights the importance of edaphic differences, but the same mechanism could work across other abiotic gradients such as altitude, rainfall, and flooding regime—wherever the impact of herbivores is dissimilar across a habitat boundary.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/305/5684/663/DC1

Abstract in Spanish

Materials and Methods

Figs. S1 and S2

Tables S1 to S3

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9 April 2004; accepted 14 June 2004

Silencing the Jasmonate Cascade: Induced Plant Defenses and Insect Populations

André Kessler, Rayko Halitschke, Ian T. Baldwin*

We transformed the native tobacco, *Nicotiana attenuata*, to silence its lipoxygenase, hydroperoxide lyase, and allene oxide synthase genes in order to inhibit oxylipin signaling, known to mediate the plant's direct and indirect defenses. When planted into native habitats, lipoxygenase-deficient plants were more vulnerable to *N. attenuata*'s adapted herbivores but also attracted novel herbivore species, which fed and reproduced successfully. In addition to highlighting the value of genetically silencing plants to study ecological interactions in nature, these results show that lipoxygenase-dependent signaling determines host selection for opportunistic herbivores and that induced defenses influence herbivore community composition.

The plant traits that are important for resistance to herbivore attack in nature are complex and operate on many spatial scales. They involve direct defenses (toxins, digestibility reducers, etc.) (1), which themselves protect plants, as well as indirect defenses, which work with components of a plant's community (natural enemies, diseases, etc.) (2–6). Moreover, plant defenses can be constitutively expressed or produced in response to an attacking pathogen or herbivore, when they are needed (7, 8).

Phenotypic plastic responses such as herbivore-induced plant defenses are thought to be an adaptation to unpredictable environments (8). In native populations of *Nicotiana attenuata* in the southwestern United States, herbivory is an unpredictable selective factor. *N. attenuata*'s ephemeral occurrence after fires (9) forces its herbivore community to

reestablish itself with every new plant population, and the plant produces a wide array of direct and indirect chemical defenses in response to this unpredictable herbivore attack (10). Many of the responses are specifically elicited by signals introduced into wounds during feeding (11), and most herbivore-induced responses studied to date require oxylipin signaling (12, 13).

Genetic transformation provides a valuable tool with which to manipulate traits that mediate complex plant-herbivore interactions and allows an integrative analysis of single traits (14–16). However, transformants are usually evaluated with known challenges, not the vast number of unknowns that occur in nature. We used transformed lines of the wild tobacco species *N. attenuata*, which express *N. attenuata* lipoxygenase 3 (*NaLOX3*), hydroperoxide lyase (*NaHPL*), and allene oxide synthase (*NaAOS*) in an antisense orientation (*as-lox*, *as-hpl*, *as-aos*, respectively) (17) to study herbivore-induced plant responses in nature. All three enzymes are key regulators in two distinct oxylipin pathways (fig.

Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Strasse 8, Jena 07745, Germany.

*To whom correspondence should be addressed. E-mail: baldwin@ice.mpg.de

S1A) and are known to play a major role in plants' wound recognition and signaling (13), but their defensive function in the rough-and-tumble of the natural environment is unknown.

In laboratory studies, plants deficient in the expression or recognition of jasmonates (JAs) derived from LOX3 are unable to elicit defense compounds and are more susceptible to herbivore attack (15, 18, 19). Similarly, silencing AOS in *N. attenuata* plants partially reduces JA and defense compound accumulation but does not attenuate the resistance to herbivores (20). HPL-derived C₆-aldehydes and -alcohols (green leaf volatiles, or GLVs) are antimicrobial and can function as direct defenses against some herbivores (21). Moreover, some C₆-compounds may function as indirect defenses (5) or play a role in eliciting defense gene expression (22) and signaling within or between plants (23). However, the function of oxylipins is not restricted to wound signaling and defense induction but includes the regulation of tuber growth (15), trichome (19) and flower development (24, 25), and ultraviolet B protection. Thus, how LOX3-, AOS-, and HPL-deficient *N. attenuata* plants would behave in their natural environment with its complexity of stressors was unknown. To extend the laboratory characterization of these genetically transformed plant lines (18, 20), we examined their growth and induced production of volatile organic compounds (VOCs), and evaluated their resistance to a specialized herbivore (*Manduca sexta*) under field conditions. We transplanted young rosette plants into the field plots and allowed them to establish for at least 1 week before experimenting (17). The growth rates [analysis of variance (ANOVA), $F_{3,118} = 1.837$, $P = 0.144$] and morphology of the three transformed plant lines did not differ from those of wild-type (WT) plants under the noncompetitive conditions of the experiment (26). The plants, however, did differ in their production of VOCs (17).

The release of herbivore-induced terpenoid compounds, in particular the sesquiterpene, *cis*- α -bergamotene, is a demonstrated sentinel for other herbivore-induced changes in oxylipin-dependent secondary metabolite production of *N. attenuata* plants (10, 17, 18). Undamaged plants of all four genotypes released equally small amounts of *cis*- α -bergamotene (ANOVA, $F_{3,11} = 0.876$, $P = 0.483$). WT plants and HPL-deficient plants released significantly increased amounts of *cis*- α -bergamotene and a suite of other terpenoid compounds after *M. sexta* damage (table S1). In contrast, the *cis*- α -bergamotene emission from herbivore-damaged *as-lox* and *as-aos* (ANOVA, $F_{3,10} = 44.56$, $P < 0.0001$; Bonferroni post hoc $P < 0.05$) plants remained low (Fig. 1A).

In addition to the open-flow VOC trapping design, we used a portable gas chro-

matograph (z-Nose) (27) to characterize the wound-induced emissions of the GLV, *cis*-3-hexenal, from WT and transformed plants (17). *As-hpl* plants released significantly less *cis*-3-hexenal than did WT, *as-aos*, and *as-lox* lines immediately after damaging the leaf tissues (ANOVA, $F_{3,19} = 33.07$, $P < 0.0001$; Bonferroni post hoc $P < 0.05$) (Fig. 1B). This demonstrates that the production of GLVs, such as *cis*-3-hexenal, requires the activity of HPL but not LOX3, which specifically supplies fatty acid hydroperoxides to the octadecanoid pathway but not to the HPL reaction (17, 18) (fig. S1). Interestingly, AOS-deficient plants had significantly higher *cis*-3-hexenal emission levels after the mechanical damage than did all other plant lines (Bonferroni post hoc $P < 0.05$) (Fig. 1B), which suggests either a rechanneling of AOS substrates into the HPL cascade (20) and/or an octadecanoid-derived negative regulation of the HPL cascade.

The altered production of secondary metabolites, such as terpenoids, in *as-lox* plants was correlated with reduced resistance to attack by the specialist herbivore *M. sexta*. Freshly hatched *M. sexta* caterpillars, which fed on rosette-stage, field-grown WT plants and similar-sized plants of the three different transformed lines (17), gained weight fastest on LOX3-deficient plants (ANOVA, $F_{3,23} = 5.83$, $P = 0.0041$). After 9 days of development on *as-lox* plants, they were 4.4-fold heavier than caterpillars on WT plants and 7-fold and 2.5-fold heavier than those on HPL- and AOS-deficient plants (Bonferroni post hoc tests, $P < 0.05$), respectively (Fig. 1C). In laboratory experiments, herbivore resistance can be restored by treating *as-lox* plants with methyl jasmonate (20). The relative resistance of AOS-deficient plants to herbivores and the susceptibility of LOX3-deficient plants confirm laboratory results and correlated with the wound-induced production of nicotine. Nicotine production was reduced only in *as-lox* plants, which was attributed to a leaky phenotype in the *as-aos* plants (fig. S1B) (17, 20). Similarly, *as-hpl* plants retain their resistance to hornworm damage despite the evidence that HPL-derived oxylipins may signal plant-defense activation (22, 23). In laboratory studies, hornworm consumption and growth were slower on HPL-deficient plants than on WT plants but could be restored to WT levels by the addition of GLVs to *as-hpl* plants, which suggested that GLVs stimulate feeding by *M. sexta* (20).

The initial field experiments showed that the three transformed plant lines (*as-lox*, *as-aos*, *as-hpl*) have similar characteristics in the field and in the laboratory (18, 20) and differ in their responsiveness to *M. sexta* attack. However, the herbivore community of *N. attenuata* is diverse and includes piercing-sucking, leaf-mining, and leaf-chewing herbivores, such as *M. sexta*. Moreover, the composition of the herbivore

community can be extremely variable and is influenced by random effects (5, 9, 10). Therefore, the exposure of genetically manipulated plants to their natural herbivore community realistically evaluates the role played by oxylipin-mediated herbivore-induced responses in structuring the plants' herbivore community.

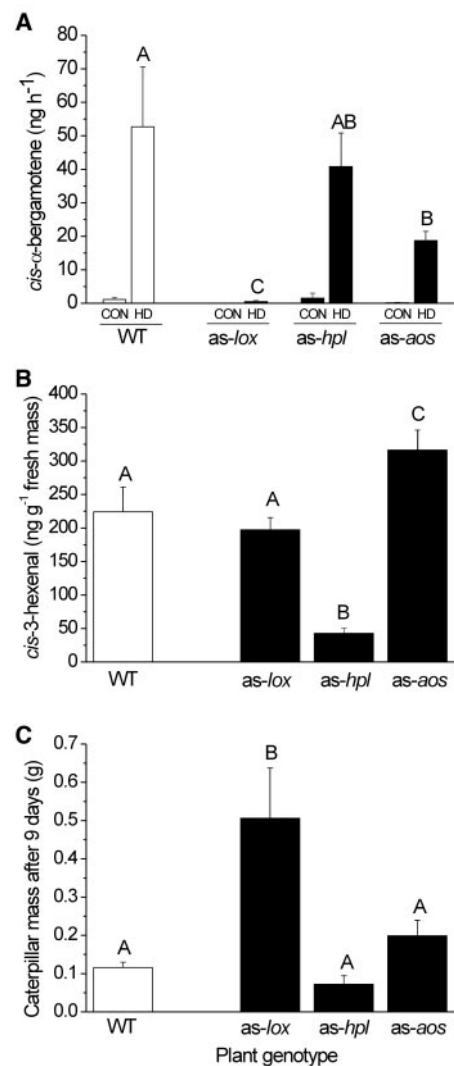


Fig. 1. Volatile organic compound emission and herbivore susceptibility of *N. attenuata* wild-type (WT) plants and plants transformed to silence LOX3 (*as-lox*), HPL (*as-hpl*), and AOS (*as-aos*) activity. (A) Mean (+SEM) *cis*- α -bergamotene emission (ng hour⁻¹) from undamaged (CON) and *Manduca sexta* hornworm-damaged (HD) plants of the four genotypes. (B) Mean (+SEM) emission of *cis*-3-hexenal (ng g⁻¹ fresh mass) measured with a portable gas chromatograph (z-Nose) in the headspace of excised leaves that had been mechanically wounded. (C) Mean (+SEM) caterpillar mass (g) after 9 days of development on WT plants compared to the genetically transformed lines. Different letters designate significantly different means as informed by a Bonferroni post hoc ($P < 0.05$) test of an ANOVA.

We transplanted 80 young rosette-stage plants in groups of four into a field plot along a linear transect (17). Each group comprised one WT plant and one plant of each of the transformed lines: *as-lox*, *as-hpl*, and *as-aos*. Extensive prior laboratory analysis of plants transformed with empty-vector constructs revealed no differences in any measured herbivore resistance trait or growth and reproduction when compared to WT plants (16). During the 2002–2003 field seasons, we observed damage from herbivores of all three feeding guilds but found very few plants that were attacked by the most damaging herbivores of previous field seasons: the leaf-chewing sphingid hornworms *Manduca quinquemaculata* and *M. sexta* and the piercing-sucking mirid bug *Tupiocoris notatus* (5, 10). Thus the observed damage, though relatively low, allowed the distribution of herbivory on the silenced lines to be clearly evaluated.

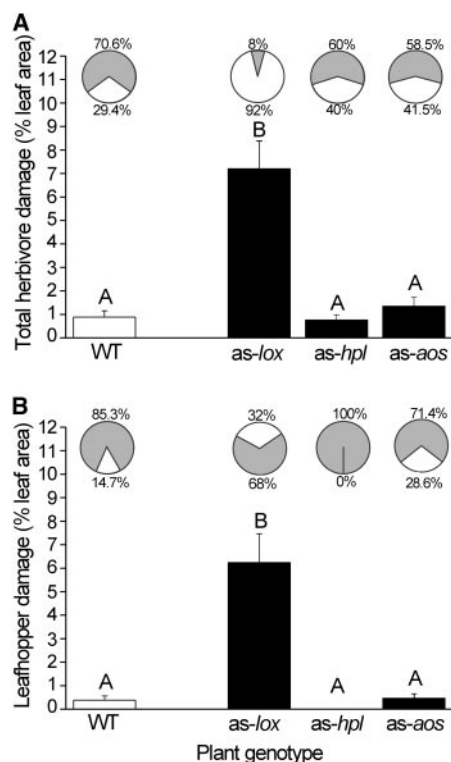


Fig. 2. Herbivore damage on field-grown *N. attenuata* wild-type (WT) plants and plants transformed to silence LOX3 (*as-lox*), HPL (*as-hpl*), and AOS (*as-aos*) activity. (A) Mean (+SEM) percentage of leaf area damaged by herbivores (bar graph) and proportion of plants in the population that were attacked by herbivores (white in the pie chart) or remained undamaged (gray in the pie chart). (B) Mean (+SEM) percentage of leaf area damaged by *Empoasca* sp. leafhoppers (bar graph) and proportion of plants in the population that were attacked by leafhoppers (white in the pie chart) or remained undamaged (gray in the pie chart). Different letters designate significantly different means as informed by a Bonferroni post hoc ($P < 0.05$) test of an ANOVA.

If plants within a population show induced defense responses to herbivory and if herbivores can freely choose among plants, herbivory should be equally distributed. Plants lacking the ability to respond to herbivore attack are more susceptible and should be preferred to responsive plants (7, 8). Consistent with this prediction, we found dramatically more herbivore damage on *as-lox* plants (ANOVA, $F_{3,120} = 24.19$, $P < 0.0001$; Bonferroni post hoc $P < 0.05$) than on WT, *as-hpl*, or *as-aos* plants (Fig. 2A, fig. S2A), which corresponds to the increased performance of *M. sexta* caterpillars on *as-lox* plants. Similarly, herbivores attacked a significantly greater proportion of *as-lox* plants compared to all other lines in the experimental population (Fig. 2A). Although herbivory was equally distributed among WT, *as-aos*, and *as-hpl* plants (Pearson chi-square test $\chi^2 < 2.518$, $P > 0.05$), it was significantly higher on plants with strongly attenuated induced responses (*as-lox*) (Pearson chi-square test $\chi^2 > 49.207$, $P < 0.0001$), which suggests that a plant's ability to elicit defenses influences the distribution of herbivory within a plant population.

Moreover, a more detailed analysis of the herbivore community revealed that the herbivore-induced plant responses can alter the host breadth of generalist herbivores. Compared to previous study years, we found two new herbivores on the experimental plants: the leafhopper *Empoasca* sp. (28) (Fig. 3, fig. S2B) and the western cucumber beetle *Diabrotica undecimpunctata tenella* Le Conte (fig. S2C). In fact, most of the observed damage on LOX3-deficient plants resulted from just one of these, *Empoasca*

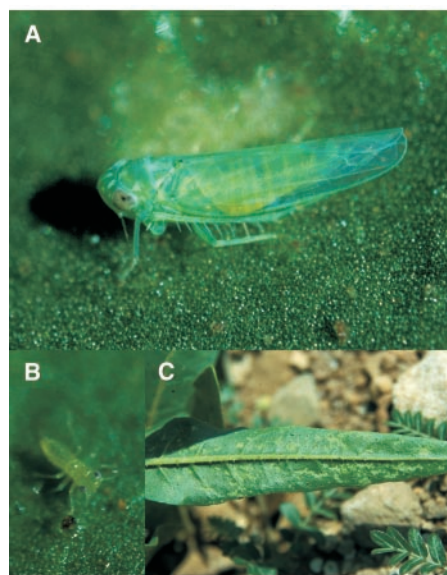


Fig. 3. *Empoasca* sp. leafhoppers on *as-lox* *N. attenuata* plants. (A) Adult *Empoasca* sp. (B) *Empoasca* sp. nymphs were exclusively found on *as-lox* plants. (C) Visual damage by *Empoasca* sp. on *as-lox* *N. attenuata* plants.

sp., which is an unusual herbivore on *N. attenuata* plants. In 12 quantitative formal surveys of 600 plants conducted over four consecutive years, we found a total of only four *Empoasca* sp. specimens on *N. attenuata* plants growing in native populations (table S2). Neither in these surveys, nor in informal observations of more than 40,000 plants over the past 15 years of field work has there been evidence of *Empoasca* feeding damage. *Empoasca* sp. is a highly mobile, opportunistic herbivore that attacks *Datura wrightii*, *Solanum americanum*, and *Mirabilis multiflora*, all of which were abundant in the study area. In our field trials with different *N. attenuata* lines, the *as-lox* plants received significantly more *Empoasca* damage than did the other transformed lines (*as-aos*, *as-hpl*) or the WT (ANOVA, $F_{3,121} = 30.25$, $P < 0.0001$, Bonferroni post hoc test $P < 0.05$) (Fig. 2B). More than 68% of the *as-lox* plants in the experimental population were damaged by *Empoasca*, whereas the proportion of damaged plants in the other lines did not exceed 29% (Fig. 2B). More importantly, *Empoasca* females oviposited on *as-lox* plants, and these plants were susceptible to attack from the emerging leafhopper offspring (Fig. 3B). *Empoasca* nymphs were found on 81% of the attacked *as-lox* plants but not at all on WT, HPL-, and AOS-deficient lines. Moreover, when given the choice (17), adult leafhoppers clearly prefer *as-lox* plants over WT plants (paired Student's *t* test, $t = -4.919$, $P = 0.0012$) (Fig. 4). Interestingly, we found no *Empoasca* damage on *as-hpl* plants, suggesting that leafhopper feeding behavior may also be influenced by GLVs, as has been demonstrated with *M. sexta* larvae (20).

The reduced resistance of LOX3-deficient plants was correlated with an altered octadecanoid-dependent gene expression in response to *Empoasca* attack. With a cDNA microarray analysis, we compared transcript accumulation in response to *Empoasca* feeding in WT and *as-lox* plants to identify

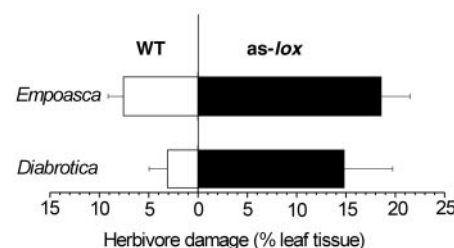


Fig. 4. Mean (+SEM) leaf tissue damage on wild-type (WT) and *as-lox* plants after 3 days of attack by *Empoasca* sp. leafhoppers or *D. undecimpunctata* leaf beetles, respectively. Ten *Empoasca* and two *D. undecimpunctata*, respectively, were allowed to choose between a WT and an *as-lox* plant that had been potted together and covered with insect mesh. Both experiments were replicated 10 times.

octadecanoid-dependent transcriptional responses (fig. S3) (17). Of the 240 *N. attenuata* genes spotted on the microarray, 54 genes showed significantly increased LOX3-dependent transcript accumulation after *Empoasca* damage and 50 showed decreased accumulation (table S3). A series of defense-related genes, such as trypsin proteinase inhibitor (TPI) and threonine deaminase, had increased expression levels, whereas others, such as xyloglucan endotransglucosylase/hydrolase or GAL83 and many of the photosynthesis-related genes (e.g., small subunit of ribulose biphosphate carboxylase), were down-regulated in response to *Empoasca* attack and showed little if any regulation in *as-lox* plants (table S3). These results suggest that a complex LOX3-dependent regulation of primary and secondary metabolism in *N. attenuata* mediates resistance to the piercing-sucking *Empoasca* leafhoppers. The fact that octadecanoid signaling plays a role in plant defense against both piercing-sucking and leaf-chewing insects suggests a common response to attack from members of these two feeding guilds. Moreover, LOX3-dependent octadecanoids may play a direct role in host-plant selection by enabling herbivores to differentiate between plants with and without intact JA signaling, as shown in experiments with the corn earworm, *Helicoverpa zea*. This generalist herbivore uses induced JA and salicylate to activate four of its cytochrome P450 genes that are associated with detoxification either before or concomitantly with the biosynthesis of allelochemicals (29).

In addition to *M. sexta*, which was affected by LOX3-mediated plant resistance traits, we found a novel leaf-chewing herbivore on *N. attenuata*, the leaf beetle *Diabrotica undecimpunctata tenella* Le Conte (fig. S2C). It often feeds on *D. wrightii* and *Cucurbita foetidissima* flowers in the study area and was observed on *N. attenuata* plants exclusively in this study and only on *as-lox* plants. To test whether or not this clear preference in the field is caused by the decreased expression of LOX3, we allowed the beetles to choose between WT and *as-lox* plants. The choice experiment revealed a clear preference for *as-lox* plants compared to WT plants (paired Student's *t* test, $t = -4.050$, $P = 0.003$) (Fig. 4).

Our results show that the LOX3-mediated inducibility of plants is crucial for the oviposition decision and for the opportunistic host selection behavior of *Empoasca* sp. and *D. undecimpunctata*, and thereby defines host breadth. Therefore, host selection seems determined not only by the plant's constitutively expressed chemical phenotype and external mortality factors (predation pressure, abiotic stress) (30) but also by the plant's ability to induce responses to herbivory. The

additional finding that induced responses to herbivory influence the distribution of herbivory within a plant community points to the value of genetically silenced plants in ecological research. An understanding of the ecological interactions that occur in nature is essential for sustainable agriculture.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1096931/DC1
 Materials and Methods
 Figs. S1 to S3
 Tables S1 to S3
 References

18 February 2004; accepted 7 June 2004
 Published online 1 July 2004;
 10.1126/science.1096931
 Include this information when citing this paper.

Osedax: Bone-Eating Marine Worms with Dwarf Males

G. W. Rouse,^{1,2*} S. K. Goffredi,^{3*} R. C. Vrijenhoek^{3†}

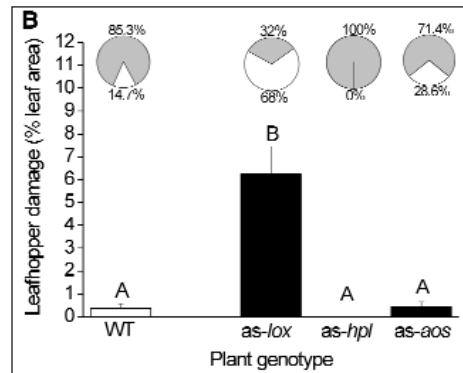
We describe a new genus, *Osedax*, and two new species of annelids with females that consume the bones of dead whales via ramifying roots. Molecular and morphological evidence revealed that *Osedax* belongs to the Siboglinidae, which includes pogonophoran and vestimentiferan worms from deep-sea vents, seeps, and anoxic basins. *Osedax* has skewed sex ratios with numerous dwarf (paedomorphic) males that live in the tubes of females. DNA sequences reveal that the two *Osedax* species diverged about 42 million years ago and currently maintain large populations ranging from 10⁵ to 10⁶ adult females.

Deep-sea exploration continues to reveal biological novelties (1) such as whale fall communities (2). Here, we describe remarkable polychaete annelids, *Osedax* gen. nov. (nov.), discovered in January 2002 on the bones of a gray whale carcass at 2891 m depth in Monterey Bay, California (3). Their conspicuous red plumes extended from most exposed portions of the whale bones (Figs. 1A and 2A). Colonies of these worms comprised two species, *Osedax rubiplumus* sp. nov. and *O.*

frankpressi sp. nov., that we describe along with the new genus. Nucleotide sequence analysis revealed that the two *Osedax* species differed by 17.28 ± 0.21% ($\bar{x} \pm SD$) for mitochondrial COI, by 7.63 ± 0.46% for mitochondrial 16S rRNA, and by 4.09 ± 0.04% for nuclear 18S rRNA (4). On the basis of a molecular clock calibrated for COI in deep-sea annelids (5), *O. rubiplumus* and *O. frankpressi* diverged about 42 million years ago (Ma) (4), in the late Eocene, when their

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Reports: "Silencing the jasmonate cascade: induced plant defenses and insect populations" by A. Kessler *et al.* (30 July 2004, p. 665). The shading of the second pie chart in Fig. 2B was reversed. The area representing the proportion of damaged plants (68%) should be displayed in white, whereas the proportion of undamaged plants (32%) should be displayed in gray, as described in the figure legend. The corrected figure is shown here.