metapodial of *Morganucodon* is only ~30%. Comparison of diaphysial diameter/bone length ratios confirms the impression that the long bones of the new haramiyid are more gracile than those of *Morganucodon*.

Among the earliest known mammals (of Late Triassic or 'Rhaeto–Liassic' age), representatives of two families, Morganucodontidae and Kuehneotheriidae, are the best known in terms of their dental apparatus. These taxa share a suite of features, including premolar–molar differentiation, multirooted post-canines (with the exception of the mesial premolars), molars with three primary cusps, slender dentaries, small dentary condyles, and unilateral, shearing occlusion[16,25,26]. *Haramiyavia clemmenseni* is comparable in the degree of premolar–molar and post-canine root differentiation. Although the condylar region of *H. clemmenseni* is not preserved, the overall shape of the slender dentary is similar to that in morganucodontids and *Kuehneotherium*; a small dentary condyle is likely to have been present. Yet significant differences separate *H. clemmenseni* from contemporaneous mammals. In *H. clemmenseni*, the sulcus anterior to the inferior alveolar foramen (Fig. 2) is proportionately wider than that in *Morganucodon* and *Kuehneotherium*, indicating that post-dentary bones were retained at a relatively more primitive, less reduced size. Furthermore, the orthal and probably bilateral occlusion produced a puncture-crushing effect that differs from the shearing mechanisms of morganucodontids and *Kuehneotherium* and the grinding dentitions of multituberculates. Thus haramiyids retain features of the jaw and post-dentary apparatus that would place them in a basal position among mammals, but at the same time they exhibit a highly specialized dentition. The dramatic dental differences exhibited by the new haramiyid in comparison to those of other Late Triassic mammals further increases the likelihood of a much earlier—possibly Middle Triassic—diversification of the earliest mammals[13]. □

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Airborne signalling by methyl salicylate in plant pathogen resistance

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Methyl salicylate, a volatile liquid, also known as oil of wintergreen, is made by a number of plants[1–9]. Here we show that methyl salicylate is a major volatile compound produced by tobacco plants inoculated with tobacco mosaic virus. Methyl salicylate is synthesized from salicylic acid, a non-volatile chemical signal required for the establishment of acquired resistance[10] and local and systemic induction of antimicrobial pathogenesis-related proteins[11]. Methyl salicylate acts by being converted back to salicyclic acid. We conclude that methyl salicylate may function as an airborne signal which acitvates disease resistance and the expression of defence-related genes in neighbouring plants and in the healthy tissues of the infected plant.

Resistance to tobacco mosaic virus (TMV) in tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc) carrying the *N* gene is associated with a hypersensitive response at sites of viral inoculation. While investigating the movement and distribution of salicylic acid (SA) in TMV-inoculated tobacco plants, we discovered that these plants produced gaseous methyl salicylic acid (MeSA) (Fig. 1). No MeSA was volatilized from healthy, mock-inoculated or mechanically wounded tobacco plants. MeSA evolution from a 5-week-old tobacco plant inoculated with TMV on two middle leaves was first observed 48 h post-inoculation and reached a maximum of 22 ng per hour per plant 84 h post-inoculation, when MeSA became one of the most abundant volatile products emitted. Increased MeSA evolution paralleled the appearance of hypersensitive lesions, which in turn coincided with the induction of SA biosynthesis[12,13]. The identification of plant-produced MeSA was confirmed by mass spectral analysis (Fig. 1c, d).

Incubation of TMV-inoculated tobacco plants at 32 °C blocks the development of the hypersensitive-resistance response and leads to systemic infection[14] without accumulation of SA or of pathogenesis-related (PR) proteins. Infected tobacco plants shifted from 32 to 24 °C undergo systemic necrosis and a massive increase in tissue SA[15]. No MeSA was detected when TMV-inoculated plants were kept at 32 °C for 48 h (Fig. 1b). When TMV-inoculated plants were shifted to 24 °C, MeSA production reached 283 ng per hour per plant 60 h after the temperature shift.

To understand the role of MeSA in the defence response, tobacco plants were preincubated for 6 days in gas-tight chambers containing air supplemented with different concentrations of MeSA. At the end of the incubation, plants were analysed for their SA content, PR-protein induction, and resistance to TMV. Incubation of tobacco plants with gaseous MeSA resulted in a dramatic accumulation of SA in the leaves (Fig. 2a). Even at 2.5 $\mu g\,l^{-1}$, MeSA increased endogenous SA content 1.4-fold compared to the untreated control, whereas 250 $\mu g\,l^{-1}$ MeSA increased SA levels 298-fold to 29.9 μg per g fresh weight. Enhanced TMV resistance, measured as reduced lesion size, was directly proportional to MeSA concentration (Fig. 2b). The mean lesion diameter was reduced by 25% by preincubation with 6.25 $\mu g\,l^{-1}$ MeSA, whereas 250 $\mu g\,l^{-1}$ of MeSA gave a 75% reduction in mean lesion diameter. Increases in tissue SA and

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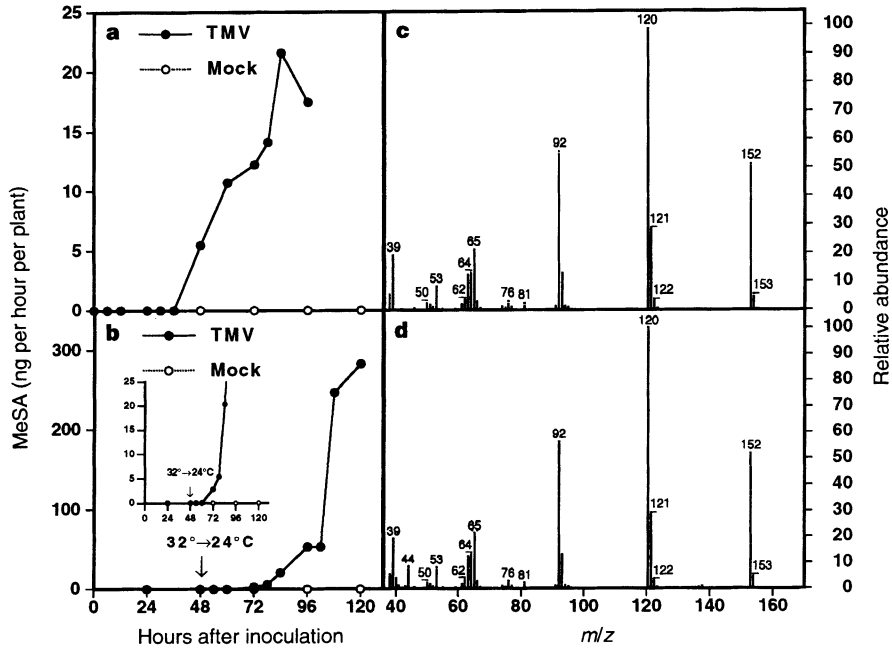
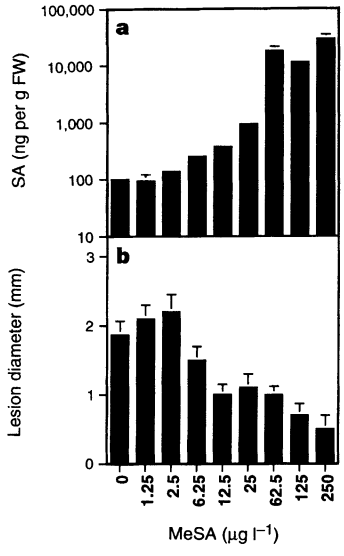


Figure 1 MeSA evolution by TMV-inoculated tobacco plants, kept at 24 °C (**a**) or incubated at 32 °C for 48 h and shifted to 24 °C at the times indicated (**b**); **b**, inset, fine scale on *y*-axis shows that no MeSA was detected in inoculated plants kept at 32 °C. Mass spectra of **c**, authentic MeSA standard, and **d**, of the putative MeSA produced by inoculated plants kept at 24 °C. The experiment was repeated three times with similar results. Note the difference in the scale of the *y*-axis in **a** and **b**.

TMV resistance following MeSA treatment was accompanied by large increases in expression of the *PR-1* gene, a molecular marker for acquired resistance (Fig. 3c). The large amounts of MeSA produced by inoculated leaves and its efficacy in inducing resistance make MeSA a likely candidate for an airborne signal which affects healthy tissues located close to the site of infection.

To establish whether the amounts of MeSA released by TMV-inoculated tobacco plants are sufficient to induce resistance in adjacent healthy plants, we constructed eight two-compartment flow-through systems (Fig. 3a). Each system consisted of a gas-tight glass chamber containing eight TMV- or mock-inoculated donor plants (D) connected to another glass chamber containing one non-inoculated recipient plant (R). Air was pumped at 30 ml min^{-1} from the donor plant chamber to the recipient plant chamber; this air passed from the donor chamber to the recipient chamber either directly (−) or through a column packed with Tenax-TA resin (+). This Tenax-TA column, which was replaced every 6 to 12 h, trapped airborne MeSA and other volatile hydrocarbons containing more than five carbon atoms[16–18]. TMV-inoculated plants were put inside the donor chamber 24 h after inoculation and maintained for 3 days. A second group of inoculated plants was then placed inside the donor chamber for another three days. A single non-inoculated plant was kept in the recipient chamber for the duration of the experiment (6 days). At the end of the experiment, total RNA was extracted from the TMV-inoculated leaves of donor plants (second group) and from healthy middle leaves of a recipient plant, and analysed by northern blotting for *PR-1* expression (Fig. 3b). In another, similar experiment eight TMV-inoculated plants kept at



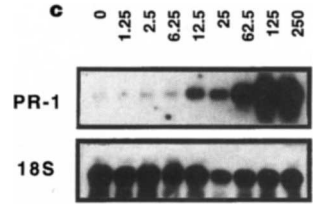


Figure 2 Effect of MeSA on **a**, total SA levels, **b**, TMV resistance, and **c**, PR-1 gene expression; FW, fresh weight. Five-week-old tobacco plants were enclosed for 6 d in 4-l gas-tight glass chambers. Different amounts of liquid MeSA were applied to the tip of a cotton swab placed inside the chamber and were allowed to evaporate. The air with MeSA was replaced every 24 h. At the end of the incubation period, one middle leaf was removed from each plant and analysed for total SA content and expression of PR genes. Plants were then inoculated with TMV on two other middle leaves, and the diameter of the TMV-induced lesions measured 4 days post-inoculation. Numbers above lanes indicate dosage of MeSA. The experiment was repeated twice with similar results.

32 °C for 3 days and then shifted to 24 °C were used as donors (Fig. 3b). Because of the longer preincubation at 32 °C, these plants showed greater necrosis and produced substantially more MeSA than plants analysed in Fig. 1b. The amount of MeSA produced by donor plants during this experiment was estimated as 184 μg (calculated by multiplying an average measured rate of MeSA production of 23 μg over 6 days by each plant, by 8—the number of donor plants). This amount of MeSA should be sufficient to activate a defence response in recipient plants (compare with Fig. 2). The temperature-shifted TMV-inoculated donor plants triggered the greatest *PR-1* expression in recipient plants. No induction of *PR-1* was detected in recipient plants when donors were mock-inoculated or when the air from TMV-inoculated plants was passed through a MeSA trap. In addition, plants receiving MeSA-containing air from TMV-inoculated plants showed a moderate increase in TMV resistance (Fig. 3c), manifesting as a statistically significant 28% reduction in lesion diameter, when compared to plants receiving air directly from mock-inoculated plants. We verified that most of the MeSA produced by donor plants was retained in the trap. No MeSA was detected in Tenax-TA traps when mock-inoculated plants were used as donors. We also verified that ethylene, a gaseous plant hormone capable of inducing some defence responses[19], is not trapped by Tenax-TA resin (data not shown). Moreover, replacing the Tenax-TA trap with a trap containing the ethylene scrubber Purafil did not prevent induction of *PR-1* in the recipient plant exposed to air from TMV-inoculated plants (data not shown).

The dependence of MeSA production on tissue SA levels, as well as the structural similarity of these compounds, indicates that MeSA in tobacco plants may be derived from SA. To investigate whether tobacco plants convert SA to MeSA, two fully expanded middle leaves of a healthy five-week-old tobacco plant were infiltrated with 1 ml of 0.5 mM ^{14}C-SA (56 mCi mmol^{-1}) and placed inside a 4-l air-tight glass chamber. Air was filtered through activated charcoal, passed through the chamber at 40 ml min^{-1}, and the volatiles collected for 12 h one day after infiltration. MeSA was trapped on a Tenax-TA column and analysed by gas chromatography and mass spectrometry (GC-MS). The mass spectrum of MeSA produced by the ^{14}C-SA-infiltrated plant showed that 99% of MeSA emitted by the infiltrated plant was derived from ^{14}C-SA (Table 1).

To determine the metabolic fate of gaseous MeSA in the tobacco leaf, a tobacco plant was incubated in air containing ^{14}C-MeSA for 48 h. The initial concentration of ^{14}C-MeSA in the 4-l air-tight glass chamber containing a single 5-week-old plant was 50 μg l^{-1}. The appearance of the molecular ion (*m/z* 140) of ^{14}C-SA in leaf extracts from ^{14}C-MeSA-treated leaves was confirmed by high-pressure liquid chromatography and liquid chromotography with mass spectrometry (LC-MS). We calculated that 93% of the SA extracted from tobacco leaves was made from gaseous ^{14}C-MeSA (Table 1).

Table 1 Methyl salicylate biosynthesis and metabolism in tobacco

Treatment	^{14}C atom % excess	Plant product	^{14}C atom % excess	Conversion (%)
^{14}C-SA (leaf infiltration)	91.67	MeSA	90.99	99.25
^{14}C-MeSA (as gas)	91.67	SA	85.51	93.28

Tobacco plants were either infiltrated into two leaves with ^{14}C-SA or gassed with ^{14}C-MeSA. ^{14}C-MeSA was prepared from 7-^{14}C SA (56 mCi mmol^{-1}; Du Pont-NEN) by methylation with diazomethane[21]. ^{14}C-labelled products (MeSA and SA) were quantified by mass spectrometry. The experiment was repeated twice with similar results.

Our data indicate that TMV-inoculated tobacco plants evolve sufficient amounts of gaseous MeSA to induce expression of PR proteins and TMV resistance in nearby healthy plants. It is known that systemic acquired resistance, in which pathogen-free parts of an infected plant become resistant to pathogen attack, depends on the systemic accumulation and possible phloem transport of SA[20,21]. We suggest that volatilization of MeSA, facilitated by tissue desiccation during necrosis, produces an airborne signal which functions as a vehicle for long-distance transport of SA within and between plants. In addition, the vascular translocation of MeSA, which is a liquid at room temperature, and benzoic acid, an immediate biosynthetic precursor of SA[21,22], may serve as back-up systems to ensure systemic accumulation of SA in the infected plant. We have observed an accumulation of large amounts of non-gaseous MeSA in TMV-inoculated leaves (data not shown). Thus, as suggested earlier[23,24], infected plants may produce other systemic signals in addition to SA. However, these signals may act by being converted to SA in target tissues. The aerial movement of MeSA also helps to explain the fact that systemic acquired resistance is observed in the leaves above and below the inoculated leaf[25].

To our knowledge, MeSA is the first airborne signal to be shown to be involved in communication between infected and healthy

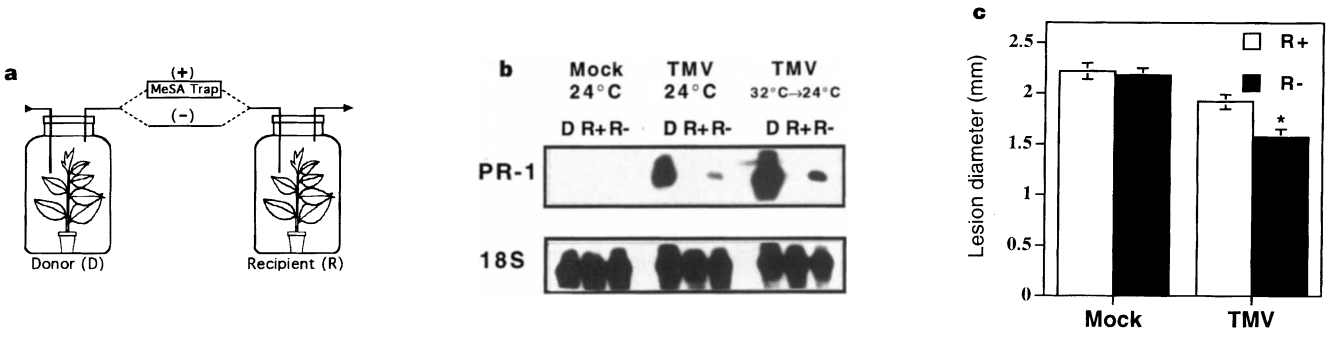


Figure 3 Induction of PR-1 gene expression and TMV resistance in healthy tobacco plants by gaseous products produced by TMV-inoculated plants. **a**, The two-compartment flow-through system. **b**, Northern blot analysis of PR-1 gene expression. Total RNA used for hybridization with a radioactive PR-1 cDNA probe was isolated from TMV- or mock-inoculated donor plants (D) and healthy recipient plants (R). **c**, Diameter of TMV-induced lesions on recipient plants 4 days after inoculation. After six days of exposure to air from donor plants, three recipient plants were inoculated on four middle leaves with TMV. The mean diameter of all lesions ± s.e. from 3 replicate recipient plants ($n = 3$) is shown. 'mock' and 'TMV' refer to the inoculation status of the donor plant; 32° → 24 °C, donor plants inoculated at 32 °C and shifted to 24 °C; + or − refers to the presence or absence of Tenax-TA trap between two chambers; asterisk indicates a statistically significant change compared with all other treatments (Student's *t*-test at $P = 0.01$). Mean lesion size in TMV (R+) treatment was not significantly different at $P = 0.01$ confidence level from either of two mock-inoculated treatments. The experiment was repeated twice with similar results.

plants. Although the significance of MeSA-mediated communication among field grown-plants is unknown, MeSA may accumulate inside dense canopies and exert a physiological effect. □

Methods

Extraction and quantification of SA. Tobacco (*Nicotiana tabacum* cv Xanthinc) seeds were germinated and grown as described[20]. Total SA (sum of free and glucosylsalicylic acid) was measured following the hydrolysis of leaf (0.5 g fresh weight) extracts with almond β-glucosidase (Sigma)[26]. SA concentrations in hydrolysed extract were determined by spectrofluorescence using HPLC[15]. All data were corrected for SA recovery.

Quantification of MeSA. Five-week-old tobacco plants were inoculated on two middle leaves with the U1 strain of TMV[20] and placed into a 1-l Wheaton purge-and-trap apparatus (Wheaton). Air filtered through activated charcoal was passed through the apparatus (40 ml per min flow rate) and volatiles were collected in a silylated glass-lined stainless-steel desorption tube (3.0 mm i.d. × 10 mm length; Scientific Instrument Services) packed with 100 mg of Tenax-TA resin (60/80 mesh; Scientific Instrument Services). D8-naphthalene was added to the absorbent traps as an internal standard. Trapped volatiles were analysed using a short-path thermal desorption system[27] (Scientific Instrument Services) connected to the injection port of a Varian 3040 gas chromatograph equipped with DB-5MS capillary column connected by a heated transfer line maintained at 280 °C to the ion source of a Finnigan-MAT 8230 high-resolution double-focusing magnetic sector mass spectrometer[21].

Liquid chromatography and mass spectrometry. LC-MS analysis was done on a Micromass Platform II LC-MS (Micromass, Altringham, UK) using negative ion atmosphere pressure chemical ionization (APCI). Cone voltage was 15 V and there was little or no fragmentation of molecular anions; source temperature was 150 °C and the APCI probe temperature was 350 °C. Data were analysed using Masslinx v.2.0 software.

RNA isolation and blot hybridization analysis. Tobacco PR-1 mRNA was detected with a radioactive probe prepared from the PR-1 cDNA by random priming (gift from E. Ward). Total leaf RNA (30 μg) was loaded in each lane and PR-1 transcripts were detected with tobacco PR-1 cDNA. As a loading control, blots were stripped and rehybridized with a probe for 18S rRNA (gift from R. Mittler).

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DNA antisense therapy for asthma in an animal model

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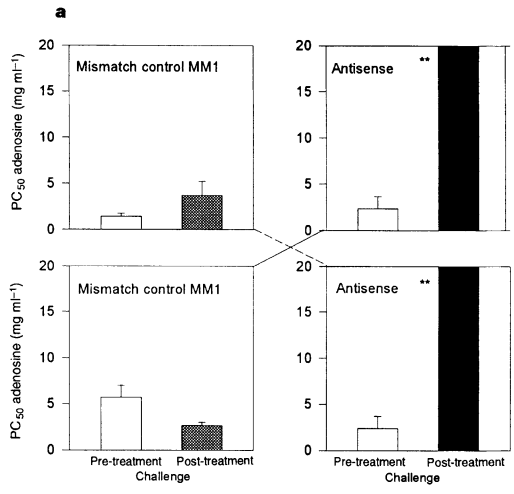
Asthma is an inflammatory disease characterized by bronchial hyper-responsiveness that can proceed to life-threatening airway obstruction. It is one of the most common diseases in industrialized countries, and in the United States accounts for about 1% of all healthcare costs[1]. Asthma prevalence and mortality have increased dramatically over the past decade[2], and occupational asthma is predicted to be the pre-eminent occupational lung disease in the next decade[3]. Increasing evidence suggests that adenosine, an endogenous purine that is involved in normal physiological processes, may be an important mediator of bronchial asthma[4–15]. In contrast to normal individuals, asthmatic individuals respond to adenosine challenge with marked airway obstruction[6,7], and concentrations of adenosine are elevated in the bronchoalveolar lavage fluid of asthma patients[9]. We performed a randomized crossover study using the dust mite-conditioned allergic rabbit model of human asthma. Administration of an aerosolized phosphorothioate antisense oligodeoxynucleotide targeting the adenosine A_1 receptor desensitized the animals to subsequent challenge with either adenosine or dust-mite allergen.



b

PC_{50} Adenosine					
A_1 MM Control		A_1 MM2 control		A_1AS	
Pre ODN	Post ODN	Pre ODN	Post ODN	Pre ODN	Post ODN
3.56 ± 1.02	3.25 ± 0.34	2.46 ± 0.50	2.81 ± 0.70	2.36 ± 0.68	>19.5 ±0.34**

Figure 1 a, Effects of adenosine A_1 receptor antisense ODN upon PC_{50} values in asthmatic rabbits. PC_{50} adenosine values were determined before and after intratracheal administration of aerosolized A_1AS or A_1MM to allergic rabbits. After a two-week rest period between parts of the experiment, rabbits were then crossed over, with those that had received A_1AS in the first part now receiving A_1MM, and those that had received A_1MM in the first part now receiving A_1AS. A_1MM2-treated animals were a separate group. **b**, Data summary. Results are presented as the mean ± s.e.m. Significance was determined by repeated-measures ANOVA and Tukey's protected *t*-test. Asterisks indicate a significant difference from all other groups, $P < 0.01$.