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Herbivory-induced volatiles elicit defence genes in lima bean leaves

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In response to herbivore damage, several plant species emit volatiles that attract natural predators of the attacking herbivores[1–5]. Using spider mites (*Tetranychus urticae*) and predatory mites (*Phytoseiulus persimilis*)[1–4], it has been shown that not only the attacked plant but also neighbouring plants are affected, becoming more attractive to predatory mites[3,6] and less susceptible to spider mites[6]. The mechanism involved in such interactions, however, remains elusive. Here we show that uninfested lima bean leaves activate five separate defence genes when exposed to volatiles from conspecific leaves infested with *T. urticae*, but not when exposed to volatiles from artificially wounded leaves. The expression pattern of these genes is similar to that produced by exposure to jasmonic acid. At least three terpenoids in the volatiles are responsible for this gene activation; they are released in response to herbivory but not artificial wounding. Expression of these genes requires calcium influx and protein phosphorylation/dephosphorylation.

We placed two lima bean (*Phaseolus lunatus* cv. Sieva) leaves in a plastic cup. Each of the leaves was infested with 100 female *T. urticae*. Within a lidded glass container, four to six lima bean leaves in a vial were kept together with the *T. urticae*-infested leaves in a plastic cup for 1 or 3 days. The *T. urticae*-infested leaves emitted the '*T. urticae*-induced' volatiles, and the leaves in the vial received the volatiles. We analysed the expression patterns of six defence genes in the leaves emitting the *T. urticae*-induced volatiles and in the leaves receiving the volatiles, and identified the volatile substances that are responsible for eliciting defence genes in the receiver leaves.

In the *T. urticae*-infested leaves, we detected the transcripts of six defence genes: genes for the basic type of pathogen-related (PR) proteins (PR-2 (β-1,3-glucanase) and PR-3 (chitinase)); an acidic type of PR-4 (chitinase); lipoxygenase (LOX) in the octadecanoid pathway; phenylalanine ammonia-lyase (PAL) in the phenylpropanoid pathway; and farnesyl pyrophosphate synthetase (FPS) related to isoprene biosynthesis (Fig. 1a). Artificial wounding of lima bean leaves elicited four of the defence genes (the acidic PR-4 and FPS genes were not elicited). We have proposed a signalling mechanism such that infestation by *T. urticae* activates both jasmonic acid (JA)- and salicylic acid (SA)-signalling pathways in lima bean leaves[7]. These compounds are known as active components of wound[8–10] or pathogen[10,11] response in higher plants. Exogenous JA or methyl salicylate (MeSA) reproduced the expression patterns of six defence genes observed in the *T. urticae*-infested leaves (Fig. 1a). The present results constitute further evidence supporting the proposed signalling mechanism.

In lima bean leaves exposed to the *T. urticae*-infested leaf volatiles for one day in the same glass container, we also detected the transcripts of five out of the six defence genes (Fig. 1a). Transcripts of the basic PR-3 and PAL genes decreased after three days. In a control experiment, lima bean leaves received the 'wound-induced' volatiles that lima bean leaves emit in response to artificial wounding. We detected only the transcript of a basic PR-2 gene in the receiver leaves. Neighbouring lima bean plants can respond to the *T-urticae*-induced and wound-induced volatiles respectively by eliciting different combinations of defence genes.

LOX in the octadecanoid pathway is a key enzyme in the biosynthesis of JA[12]. Enzymatic activity of LOX in lima bean leaves that received the *T. urticae*-induced volatiles increased as high as the activity in the *T. urticae*-infested leaves (Table 1). Previous treatment with a LOX inhibitor, SHAM, completely blocked the expression of basic PR-2, basic PR-3, LOX, PAL and FPS genes in the receiver leaves (Fig. 1b). This inhibitory effect was overcome by simultaneous treatment of exogenous JA with the inhibitor. In the receiver leaves, the *T. urticae*-induced volatiles might induce *de novo* synthesis of LOX followed by biosynthesis of JA, which subsequently activates a subset of defence genes.

Treatment with a chelator of extracellular Ca^{2+} ion, BAPTA, or an inhibitor of serine/threonine protein kinases, staurosporine, completely suppressed the expression of the five defence genes in lima bean leaves that received the *T. urticae*-induced volatiles (Fig. 1b). An inhibitor of type 1 and/or type 2A protein phosphatases, calyculin A, suppressed the expression of basic PR-3, PAL and FPS genes completely, but that of basic PR-2 and LOX genes only slightly. Therefore, the signalling pathway(s) mediating expression of defence genes in the receiver leaves would include the calcium influx into cells, protein phosphorylation, and dephosphorylation steps.

Table 1 Inductions of LOX activity in lima bean leaves

Duration (days)	Enzyme activity (relative activity*)		
	Control	Emitter leaves infested by *T. urticae*	Receiver leaves
0	1.43 ± 0.24 (1.00)	–	–
1	–	2.18 ± 0.33 (1.52)	6.95 ± 1.55 (4.87)
3	1.29 ± 0.19 (0.94)	5.08 ± 1.41 (3.57)	3.80 ± 0.35 (2.66)

*Relative to the activity of the control experiment at day 0. Lima bean leaves were either infested with *T. urticae* or receiving the *T. urticae*-induced volatiles. Values are means ± standard deviation of three experiments (ΔA_{234} per mg protein per min).

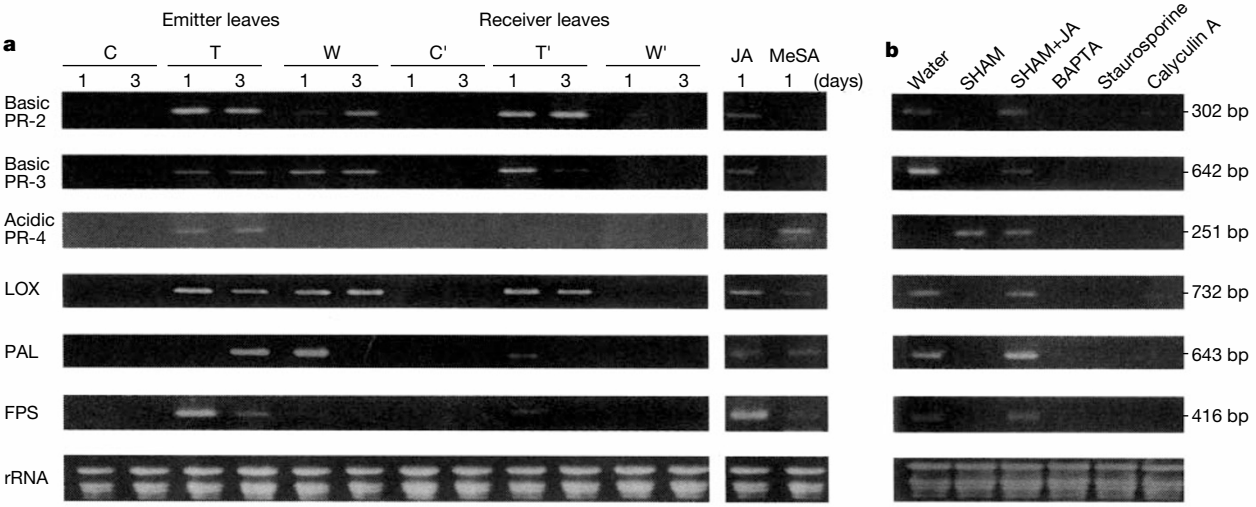

Figure 1 Expression of defence genes in lima bean leaves. **a,** Receiver leaves (C′, T′ and W′) were exposed to volatiles from emitter leaves with the same symbols: uninfested (C), *T. urticae*-infested (T) or artificially wounded (W) leaves. An aqueous jasmonic acid (0.1 mM; Wako, Japan) was applied from the petioles of leaflets (JA). Methyl salicylate (Wako) was applied to leaves as a gas (about 30 p.p.b.) in a glass bottle (2 litre) (MeSA). **b,** Leaves were treated with salicylhydroxamic acid (SHAM, 10 mM; Sigma, USA), SHAM + JA (0.1 mM), 1,2-*bis*-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA, 5 mM; Sigma), staurosporine (2 μM; Wako, Japan), or calyculin A (3 μM; Sigma), through the petioles of leaflets. Three hours after each application, the leaves were placed in a glass container together with *T. urticae*-infested leaves for one day. bp, base pairs.

Major chemical components from the *T. urticae*-infested leaf volatiles are (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (Fig. 2). Concentrations of these substances increased with the gradual infestation by *T. urticae* over 24 h. In the volatiles, we did not observe enhancement in the emission of linalool and MeSA which had been reported as chemical components in *T. urticae*-induced lima bean leaf volatiles[4]. The major chemical component of the wound-induced volatile was (*Z*)-3-hexenol. Emission of this substance was transient. Terpenoids and MeSA were scarcely detected in the wound-induced volatiles for 24 h after the wound was made. Such temporal, quantitative and qualitative differences in chemical components between the *T. urticae*-induced and the wound-induced volatiles might induce the different expression patterns of defence genes in receiver leaves.

Airborne methyl jasmonate (MeJA) and MeSA were proposed to mediate plant–plant interactions in some specific cases[8,13]. In the *T. urticae*-induced volatiles, however, we detected no MeJA but 2.97 ± 0.42 μg MeSA per gram fresh weight per hour. This was as low as 2.71 ± 0.48 μg MeSA per gram fresh weight per hour in the volatiles from the uninfested lima bean leaves in a control experiment. In addition, no transcript for an acidic PR-4 gene associated with MeSA was detected in the receiver leaves (see Fig. 1). Thus, MeSA is detectable in the *T. urticae*-induced volatiles but is less likely to be an active component in mediating the plant–plant interactions in the present study.

We exposed uninfested lima bean leaves to the vapours of chemically synthesized preparations of four chemicals and analysed transcripts of defence genes in the leaves (Fig. 3). DMNT and TMTT induced the expression of basic PR-2 and PR-3, LOX, PAL and FPS genes in the leaves within 3 h of exposure. Likewise, β-ocimene induced the expression of basic PR-2 and PR-3, PAL and FPS genes in 24 h. Linalool, in contrast, induced no expression for the six defence genes we analysed here. DMNT, TMTT, and β-ocimene in the *T. urticae*-induced volatiles are responsible for the induction of defensive responses in the receiver leaves. Another potential ingredient that is not detectable by the present analytical method is ethylene. Tobacco plants emit ethylene in response to herbivory[14]. Another two potential ingredients are (*Z*)-3-hexenol and

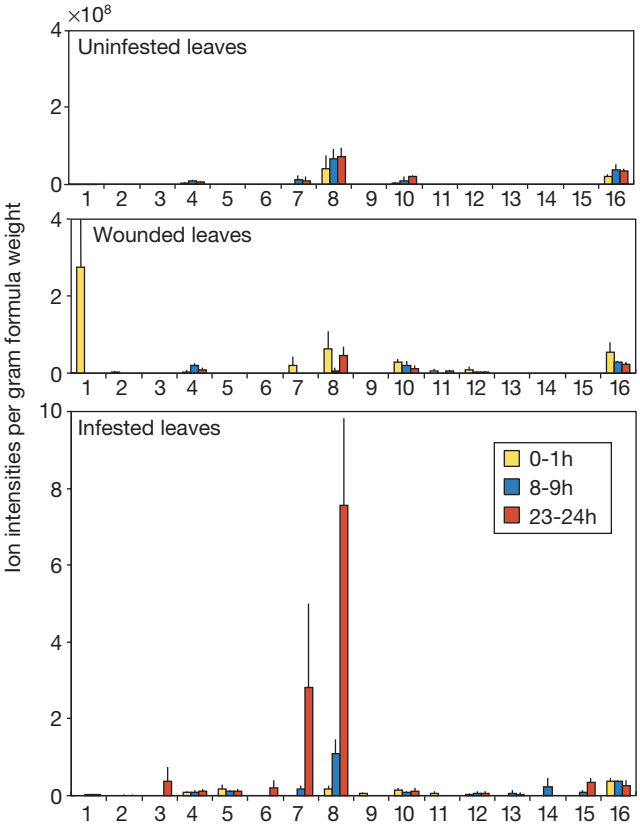

Figure 2 Compounds identified in headspace volatiles of detached leaves. The two leaves in each treatment were placed in a lidded glass container for 24 h, except for when volatile collections were performed at 0–1 h, 8–9 h and 23–24 h of the experimental period. The collected volatiles were subjected to GC-MS analysis ($n = 3$). 1, (*Z*)-3-hexenol; 2, (*E*)-2-hexenal; 3, (*Z*)-3-hexenyl acetate; 4, α-pinene; 5, limonene; 6, (*Z*)-β-ocimene; 7, (*E*)-β-ocimene; 8, DMNT; 9, menthol; 10, α-copaene; 11, junipene; 12, β-caryophyllene; 13, α-humulene; 14, germacrene d; 15, TMTT; 16, methyl salicylate.

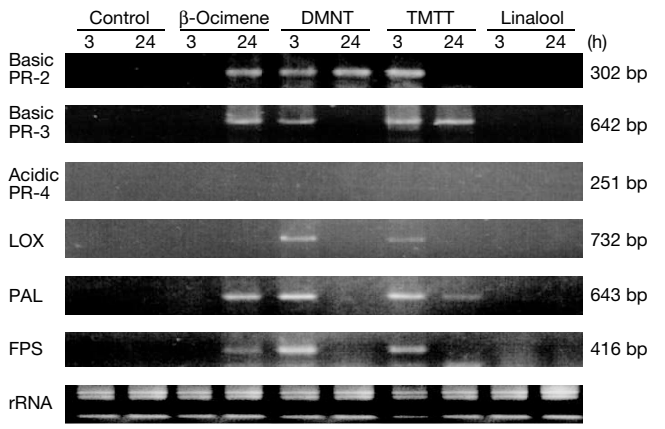

Figure 3 Expression of genes in leaves fumigated with a terpenoid compound. We enclosed uninfested leaves in a glass container together with a piece of cotton wool containing a chemical (β-ocimene, DMNT, TMTT, and linalool) dissolved in dichloromethane for 3 or 24 h. A piece of cotton wool containing dichloromethane was the control.

(*Z*)-3-hexenyl acetate. These are responsible for the defensive responses in *Arabidopsis* plants[15].

We evaluated how suitable the lima bean leaves receiving the *T. urticae*-induced volatiles are as a food resource for *T. urticae*. We exposed lima bean leaves to the *T. urticae*-induced volatiles for three days then infested them with 100 female *T. urticae* per leaf for another three days ('sample' leaves). We measured the loss of chlorophyll in the infested leaves as an indication of consumption by *T. urticae*[16]. In the control experiment, we exposed lima bean leaves to the volatiles from uninfested leaves then infested them with the same number of female *T. urticae* ('control' leaves). The amounts of chlorophyll in the sample and the control leaves were 0.56 ± 0.16 and 0.56 ± 0.01 mg per gram fresh weight without infestation, respectively. After being infested by *T. urticae*, the amounts of chlorophyll from the sample and control leaves were 0.48±0.02 and 0.42±0.02 mg per gram fresh weight, respectively. The chlorophyll lost from the sample leaves was significantly lower than that from the control leaves. Between infestation states, $F_{1,32}$ = 50.083 and $P < 0.0001$; between volatiles, $F_{1,32}$ = 4.579 and P = 0.0401; and the interaction of the infestation and the volatiles, $F_{1,32}$ = 6.580 and P = 0.0152 (ANOVA). Thus, after exposure to the *T. urticae*-induced volatiles, lima bean leaves become less suitable as a food resource to *T. urticae*.

The present analyses suggest the following mechanism of plant–plant interactions among lima bean plants that are mediated by airborne signals. In response to spider mites, a lima bean plant induces expression of at least six defence genes and emits volatiles accordingly. After plants are exposed to the terpenoids in the volatiles, a LOX gene is induced in neighbouring plants, followed by activation of the JA signalling pathway, and then expression of multiple defence genes that JA mediates. These plants can prepare defences against the spider mites in advance. The *T. urticae*-induced volatiles are specific and different in chemical composition from the wound-induced volatiles. Neighbouring plants can recognize such specificity and differences between leaf volatiles.

These findings on airborne information transferred among plants are important for understanding multitrophic interactions in ecosystems as well as for developing new methods for plant protection against herbivores. □

Methods

Bioassay and chemical application

Throughout our experiments, we detached a primary leaf with the petiole from intact lima bean plants (2 to 3 weeks old) and immediately transferred it into a vial (6 ml) containing distilled water. We then placed about 100 *T. urticae* females on the detached leaf. Two infested leaves were placed in a plastic cup (10-cm diameter, 5.5-cm depth). To prevent the escape of *T. urticae* from the infested emitter leaves, we lined the inner wall of the cup with wet cotton wool. Artificially wounded leaves were also prepared by punching 35 holes (5-mm diameter) into each leaf. We placed the cups holding the infested or artificially wounded leaves in a lidded glass container (7 litre) together with four to six uninfested receiver leaves set in vials containing water or chemical solution(s). The experimental setup was maintained at 25 ± 2 °C, under conditions of 50% to 70% relative humidity and 16 hours of light/8 hours of darkness for 1 or 3 days. We took care to ensure that the receiver leaves were never infested by *T. urticae*.

β-ocimene, DMNT and TMTT were synthesized in the laboratory. Linalool was obtained from Tokyo Chemical Industry. The chemicals were dissolved in pure dichloromethane (μg μl^{-1}). Each chemical (10 μl) was impregnated into a piece of cotton wool. After evaporation of the solvent, we enclosed the piece of cotton wool in a container (7 litre) together with two uninfested receiver leaves for 3 or 24 h under the conditions described above. All chemicals, except β-ocimene, were more than 98% pure. β-ocimene was a mixture of *E* (about 70%) and *Z* (about 30%) isomers.

RT–PCR (polymer chain reaction after reverse transcription of RNA) analysis

Total RNA was extracted from two leaves by means of the acid guanidinium-phenol-chloroform method[17]. First-strand cDNA was synthesized from 1 μg of total RNA using a primer (5′-CTTGCACCATCTATCTCTTC-3′, 5′-GATGCGGTCTTGAACCCTGC-3′, 5′-GGACTCTTTTGATTCTCATC-3′, 5′-TGACAATATCTCTATGACTG-3′ or 5′-TCCT CCAAATGCCTCAAGTC-3′), which corresponded, respectively, to nucleotides 336 to 355, 881 to 900, 864 to 883, 1972 to1991, or 937 to 956 in the sequence of *Phaseolus vulgaris* acidic chitinase[18], basic chitinase[19], basic β-1,3-glucanase[20], pLOX3[21] or PAL cDNA[22]. The first-strand cDNA was amplified by adding Taq DNA polymerase (Takara, Japan) and a further primer (5′-AGCAACAACGTTAATGTTGC-3′, 5′-CTCAGCGCCC TCATATCC AG-3′, 5′-TATGCTCTTTTCACTTCACC-3′, 5′-CTAGCAACAAACAGGCAACT-3′ or 5′-AGGCTGCTGCCATTATGGAG-3′), which corresponded, respectively, to nucleotides 105 to 124, 259 to 278, 582 to 601, 1260 to 1279, or 314 to 333 in the sequences of *P. vulgaris* acidic chitinase, basic chitinase, basic β-1,3-glucanase, LOX, or PAL cDNA in those reports. Synthesis consisted of 30, 25, 23, 23 or 25 cycles (under optimum dynamic ranges before arriving at the plateau) of 95 °C for 40 s, 55 °C for 90 s and 72 °C for 60 s for the amplifications of acidic chitinase, basic chitinase, basic β-1,3-glucanase, pLOX3, and PAL cDNA, respectively. FPS cDNA was amplified with a pair of primers, 5′-CATGGATGAC TCTCACACTC-3′ and 5′-AGTCATCCTGGACTTGAAAG-3′ which corresponded, respectively, to nucleotides 405 to 424 and 801 to 820 in the sequence of maize FPS cDNA[23] during 35 cycles (at 95 °C for 40 s, 55 °C for 90 s and 72 °C for 1 min), after the first-strand cDNA was synthesized using a random nonamer primer. An equal amount of PCR products and 5 μg of total RNA were separated by electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide. No PCR products were amplified without reverse-transcription reactions, and the sequence analysis confirmed that each cDNA product was amplified from the transcribed product of lima bean gene.

Enzyme assay

We prepared enzyme extract by homogenization of a leaf with a grinding buffer containing 100 mM sodium phosphate (pH 7.0), 7% polyvinylpolypyrrolidone and 1% Triton X-100, followed by centrifugation. We measured the LOX activity[24] and protein concentrations[25] in the supernatant.

Analysis of volatile compounds

We collected the volatile compounds emitted from the two leaves in a lidded glass bottle (2 litre) using a glass desorption tube (3.0 mm i.d. × 160 mm length) packed with 100 mg of Tenax-TA resin (20/35 mesh; GL sciences, Japan) at a 100 ml min^{-1} flow rate for 1 h. The absorbed compounds were eluted with 2 ml of diethyl ether, and then *n*-eicosane was immediately added to the eluate as the internal standard. After the eluate was concentrated with a stream of gaseous N_2, it was injected into an injection port (250 °C) of a gas chromatograph-mass spectrometer (GC-MS) (GC: Hewlett Packard 6890 with an HP-5MS capillary column with 0.25 mm i.d., 30 m length, 0.25 μm film thickness. MS: Hewlett Packard 5973 mass selective detector, 70eV). The GC oven temperature was programmed to rise from 40 °C (5 min. hold) to 280 °C at 15 °C min^{-1}.

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Genetic and epigenetic mechanisms contribute to motor neuron pathfinding

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Many lines of evidence indicate that genetically distinct subtypes of motor neurons are specified during development[1], with each type having characteristic properties of axon guidance and cell-body migration[2]. Motor neuron subtypes express unique combinations of LIM-type homeodomain factors that may act as intrinsic genetic regulators of the cytoskeletal events that mediate cell migration, axon navigation or both[3–7]. Although experimentally displaced motor neurons can pioneer new routes to their targets[8–11], in many cases the axons of motor neurons in complete isolation from their normal territories passively follow stereotypical pathways dictated by the environment[12–16]. To investigate the nonspecific versus genetically controlled regulation of motor connectivity we forced all motor neurons to express ectopically a LIM gene combination appropriate for the subgroup that innervates axial muscles. Here we show that this genetic alteration is sufficient to convert the cell body settling pattern, gene-expression profile and axonal projections of all motor neurons to that of the axial subclass. Nevertheless, elevated occupancy of the axial pathway can override their genetic program, causing some axons to project to alternative targets.

At the birth of motor neurons the genes *Isl1*, *Lhx3* (*Lim3*) and the redundant gene *Lhx4* (*Gsh4*) act together to direct axons ventrally from the neural tube[17]. This early combinatorial pattern of LIM gene expression changes rapidly as motor axons emerge ventrally from the neural tube, so that each ventral-exiting motor column subtype expresses a distinct combination of factors (Fig. 1). To examine whether LIM homeodomain transcription factors define motor column identity we altered their combinatorial pattern of expression in developing motor neurons. We sought to convert all motor neurons to an axial-innervating (medial half of median motor column, MMCm; Fig. 1) identity by ectopically expressing Lhx3, a LIM homeodomain factor specific to the MMCm subtype. We used the promoter of the motor-neuron-specific gene *HB9* (refs 18, 19) to drive expression of *Lhx3* (Fig. 2a). In two control mouse lines we expressed nuclear-localized β-galactosidase (nLacZ) or the LMC-motor neuron LIM homeodomain factor Lhx1 (Lim-1) using the same strategy (Fig. 2a). We used a Cre-removable lox–neomycin–lox (LNL) block on transgene translation to avoid the expected lethality of severe motor defects. One copy of each

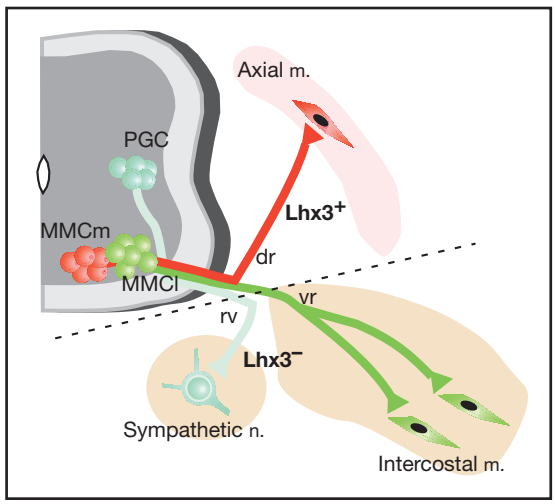

Figure 1 Summary of thoracic motor columns. Motor columns express unique combinations of LIM homeodomain transcription factors which emerge from earlier patterns of LIM factors at motor neuron birth[17]. MMCm cells (red) express Isl1, Isl2, Lhx3, Lhx4 and HB9. Lhx3 and Lhx4 have redundant activities[17], therefore only Lhx3 is shown. MMCm motor neurons project axons to axial muscles through the dorsal ramus (dr) pathway at thoracic levels. At lower cervical levels the axial musculature is more complex and MMCm motor neurons use several branches to innervate these targets. MMCl cells (green) project axons into the ventral ramus (vr) and express Isl1, Isl2 and HB9. PGC cells (blue) project axons into the ramus visceralis (rv) and express Isl1 and HB9. At limb levels (lower cervical and lumbar) LMC motor neurons (Isl1, Isl2, Lhx1, HB9) occupy a position similar to the PGC in the spinal cord and project axons along a ventral ramus-like pathway to plexi at the base of the limb. The spinal cord at limb levels does not contain PGC or MMCl cells. MMCm, medial half of median motor column; MMCl, lateral half of median motor column; PGC, preganglionic motor column; LMC, lateral motor column; m, muscle; n, neuron.

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