Transgenic N. glauca plants expressing bacterial virulence gene virF are converted into hosts for nopaline strains of A. tumefaciens

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from the Devonian such as *Aglaophyton*, *Rhynia* or *Asteroxylon*, which contain structures interpreted to be vesicles and spores resembling those formed by extant *Glomus*[11]. Our dating of the origin of VAM fungi is consistent with the hypothesis that symbiotic fungi were instrumental in the colonization of land by ancient plants[15]. This hypothesis is also supported by the observation that endomycorrhizae can now be found worldwide on most extant plant families, in the angiosperms, gymnosperms as well as vascular cryptogams (ferns), suggesting that its nature is ancestral. □

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1. Knoll, A. H. *Science* **256,** 622–627 (1992).
2. Morton, J. B. *Mycotaxon* **37,** 493–515 (1990).
3. Morton, J. B. & Benny, G. L. *Mycotaxon* **37,** 471–491 (1990).
4. Morton, J. B. *Mycologia* **82,** 192–207 (1990).
5. Gederman, H. *A. Rev. Phytopath.* **6,** 397–418 (1968).
6. Mosse, B. *A. Rev. Phytopath.* **11,** 171–196 (1973).
7. Pérez, Y. & Schenck, N. C. *Mycologia* **82,** 256–260 (1990).
8. Trappe, J. M. in *Ecophysiology of VA Mycorrhizal Plants* (ed. Safir, G. R.) 5–25 (CRC, Boca Raton, 1987).
9. Bruns, T. D. *et al. Molecular Phylogenetics and Evolution* **1,** 231–241 (1992).
10. Stubblefield, S. P., Taylor, T. N. & Trappe, J. M. *Am. J. Bot.* **74,** 1904–1911 (1987).
11. Pirozynski, K. A. & Dalpé, Y. *Symbiosis* **7,** 1–36 (1989).
12. Li, P. & Bousquet, J. *Molec. Biol. Evol.* **9,** 1185–1189 (1992).
13. Wolfe, K. H., Gouy, M., Yang, Y. W., Sharp, P. & Li, W. H. *Proc. natn. Acad. Sci. U.S.A.* **86,** 6201–6205 (1989).
14. Stewart, W. N. *Paleobotany and the Evolution of Plants* (Cambridge University Press, Cambridge, 1983).
15. Pirozynski, K. A. & Malloch, D. W. *BioSystems* **6,** 153–164 (1975).
16. Simon, L., Lalonde, M. & Bruns, T. D. *Appl. envir. Microbiol.* **58,** 291–295 (1992).
17. Swofford, D. L. *PAUP-Phylogenetic Analysis Using Parsimony* (Illinois Natural History Survey, Champaign, 1990).
18. Jukes, T. H. & Cantor, C. R. in *Mammalian Protein Metabolism* (ed. Munro, H. N.) 21–132 (Academic, New York, 1969).
19. Saitou, N. & Nei, M. *Molec. Biol. Evol.* **4,** 406–425 (1987).
20. Kimura, M. *J. molec. Evol.* **16,** 111–120 (1980).

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Transgenic *N. glauca* plants expressing bacterial virulence gene *virF* are converted into hosts for nopaline strains of *A. tumefaciens*

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TUMOURS are induced by *Agrobacterium tumefaciens* on a variety of plants[1–3]. The virulence determinants of *A. tumefaciens* reside on a large tumour-inducing (Ti) plasmid. This plasmid carries two regions essential for tumour induction, namely the T region and the Vir region. During infection the T region is transferred to the plant cell, where it becomes stably integrated in one of the host chromosomes as T-DNA. Expression of T-DNA leads to the production of the plant hormones auxin and cytokinin, as well as to the synthesis of specific amino-acid derivatives termed opines. *Agrobacterium* strains are classified according to the types of opines produced by the tumours they induce. The Vir region contains genes that are expressed in the bacterium and are required for T-DNA transfer to plant cells, and several other genes that affect the efficiency of transfer and the host range. Vir regions from different Ti plasmids may vary slightly in the genes they contain: for instance, the *virF* gene, which is present in the Vir-region of octopine Ti plasmids, is absent from nopaline Ti plasmids[4,5]. Mutation of the *virF* gene leads to a weakened virulence of octopine strains on tomato[6] and *Nicotiana glauca* (shrub tobacco)[4]. Nopaline strains are strongly attenuated in *N. glauca* compared with octopine strains because of the absence of the *virF* virulence gene from the Ti plasmid in nopaline strains[4]. The *virF* gene product may be transferred to and be active in plant cells. Here we isolate transgenic *N. glauca* plants in which the *virF* coding sequence is expressed using the cauliflower mosaic virus 35S promoter. The presence of the VirF protein converts the non-host *N. glauca* into a host for tumour formation by *A. tumefaciens* nopaline strains and octopine *virF* mutants. Our results indicate that certain virulence gene products such as the VirF protein may be transferred to plant cells during tumour induction, where they function as mediators of T-DNA transfer.

In contrast to other *vir* mutants, strains defective in *virE* or *virF* can be complemented for tumorigencity by coinfection with a helper strain such as LBA 4404, which does not carry a T region and so is not oncogenic, but which has an intact octopine-type Vir region[5,7]. Helper strains in which one of the essential *vir* genes is defective cannot complement *virE* and *virF* mutants for tumour induction[4,7], indicating that a complete *vir*-encoded (T-DNA) transport system is necessary for complementation by co-infection. As there is no T-DNA transfer from these helper strains, certain Vir proteins or their products may be transferred to plant cells by means of this *vir*-encoded

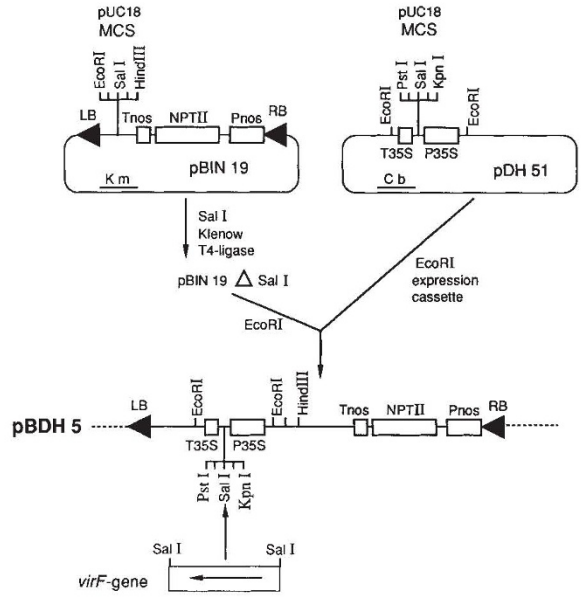

FIG. 1 Construction of a vector for expressing the VirF protein in plants. The plant expression vector pBDH5 present in *E. coli* CEL475 (ref. 13) was constructed by deleting the *Sal*I restriction site from pBIN19 (ref. 14) and subsequently inserting a CaMV 35S expression cassette from pDH51 (ref. 15) into the *Eco*RI site. To clone the coding region of the *virF* gene as a *Sal*I fragment in pBDH5, it was first subcloned as a *Bsp*HI fragment from plasmid pRAL7007 (ref. 4), which is a derivative of pIC19R (ref. 16) with an *Sst*I fragment containing the *virF* gene, into the *Nco*I site of pMTL24 (ref. 17). The resulting construct was termed pRAL7014. Plasmid pRAL7014 was isolated from *E. coli* and electroporated into the *A. tumefaciens* helper strain LBA4404 (ref. 18). Cloning procedures were according to ref. 19; electroporation of agrobacteria was according to ref. 20. Abbreviations: MCS, multiple cloning site; LB, RB, left and right T-DNA border repeats; T35S and Tnos, cauliflower mosaic virus (CaMV) 35S and nopaline synthase terminator sequences; P35S and Pnos, CaMV 35S and nopaline synthase promoter sequences; NPTII, neomycine phosphotransferase gene; Km, bacterial kanamycin-resistance selection marker; Cb, bacterial carbenicillin-resistance selection marker.

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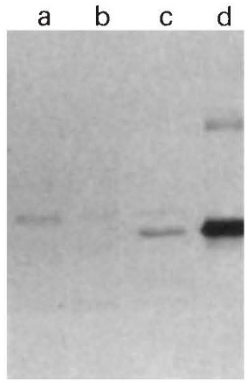

FIG. 2 Presence of the VirF protein detected in transgenic *N. glauca* plants. Plant extracts were screened for the presence of the VirF protein by immunoblotting with antiserum raised against VirF from *E. coli*. Lane a, wild-type *N. glauca*; lane b, non-transgenic F1 *N. glauca* seedling; lane c, transgenic F1 *N. glauca* seedling; lane d, 25 ng VirF protein purified from *E. coli*.
METHODS. Leaf material (200 mg) was powdered in liquid nitrogen and boiled for 5 min with 1 ml Laemmli buffer[21]. A volume of 20 µl (~5 µg) protein extract was separated by electrophoresis on a 12.5% SDS–polyacrylamide gel. Visualization of the VirF band by immunoblotting (western blot) was as described in ref. 22. VirF antiserum was used in a 2,000-fold dilution. Bands were stained using the alkaline phosphatase reaction according to the Protoblot immunoscreening system (Promega Biotec).

transport system or be secreted through a default pathway in the absence of plant cells. To investigate this idea, we used antibodies raised against the VirF protein to detect VirF in different cell fractions. Most was found in the cytoplasm and some in the membrane fraction, but none was detected in the medium (our unpublished results). Tumour formation by *virF* mutants on *N. glauca* was not stimulated by addition of VirF protein or the *vir*-induction medium used to grow *virF*$^+$ strains. On the other hand, oncogenicity of wild-type strains on *N. glauca* was not diminished by addition of VirF antibodies. Although not conclusive, these experiments indicate that VirF may itself be transported to plant cells during tumour induction by the *vir*-encoded transport system and then promote T-DNA transfer from *virF* mutants to these plant cells.

We constructed transgenic *N. glauca* plants expressing VirF protein (Fig. 1). Seedlings of these transgenic plants (obtained after crossing with wild type) were infected with *A. tumefaciens* strains by stem inoculation (Figs 2, 3). Octopine strains carrying a *virF* gene formed tumours equally well on *virF*$^+$ and *virF*$^-$ plants. Nopaline strains, which lack the *virF* gene, and *virF* mutants were avirulent on non-transgenic *N. glauca*, but were as virulent as octopine strains on the transgenic *virF*-expressing *N. glauca* plants. Wounding or infection with a helper strain lacking a T region (LBA1115), a *virB* mutant (LBA1512) or a *virE* mutant (LBA1514) did not result in a tumour (Fig. 4). Therefore expression of the bacterial *virF* gene in transgenic *N. glauca* plants converts this non-host into a host for tumour induction by nopaline strains of *A. tumefaciens*.

To find out whether the increased susceptibility for tumour formation of *virF*$^+$ *N. glauca* plants was due to a more efficient T-DNA uptake or to an increased sensitivity for the phytohormones produced as a result of the T-DNA, we did leaf disc transformation experiments with strains containing the binary vector pGUS-Int, which has the 'GUS-intron' reporter gene between the border repeats[8]. Leaf discs of *virF*$^-$ and *virF*$^+$ *N. glauca* were infected with a wild-type octopine strain and the *virF* mutant LBA1517 (ref. 6). Transfer of T-DNA (revealed after histochemical staining for β-glucuronidase) from the wild-type strain was equally efficient to leaf cells from *virF*$^-$ and *virF*$^+$ plants. However, transfer of T-DNA from the *virF* mutant occurred at a significantly higher frequency to leaf cells from *virF*$^+$ plants than to those from *virF*$^-$ plants (not shown). This demonstrates that the presence of the VirF protein in the transgenic plants leads to more efficient T-DNA uptake rather than to increased susceptibility to phytohormones.

Our results show that the VirF protein is active in mediating T-DNA transfer from *Agrobacterium* to plant cells even when expressed from a plant promoter in the plant cell. Co-inoculation experiments indicate that the VirF protein is transferred from *Agrobacterium* to plant cells during tumour induction by means of the *vir*-encoded transport system, but to verify this hypothesis more evidence is needed. At least one protein in the *vir* system is homologous to certain proteins that participate in the systems mediating protein secretion from Gram-negative bacteria[9,10]. As the *vir*-encoded transport system mediates conjugation between bacteria[11,12], the transport systems mediating transfer of DNA

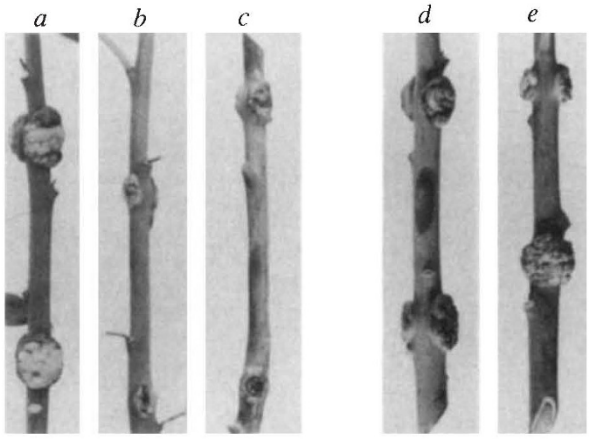

FIG. 3 Tumour response on wild-type (*a*–*c*) and kanamycin-resistant *virF*-expressing transgenic *N. glauca* seedlings (*d*, *e*) after infection with different *Agrobacterium* strains. *a*, Infection with octopine strain LBA1010 (ref. 23); *b*, with *virF* mutant LBA1517 (ref. 6); *c*, with nopaline strain LBA958 (ref. 24); *d*, with *virF* mutant LBA1517; *e*, with nopaline strain LBA958.
METHODS. Transgenic *N. glauca* plants were obtained by the leaf disc transformation method[25] which was modified as follows. Surface-sterilized *N. glauca* leaf discs were dipped in an overnight culture of a derivative of *A. tumefaciens* strain LBA4404 with plasmid pRAL7014, blotted dry on filter paper and placed on solid SM-O mecium (MS agar containing 0.03 mg l^{-1} indole acetic acid and 1 mg l^{-1} kinetine). After 3 d the leaf discs were transferred to solid SM-O plates containing 100 mg l^{-1} cephotaxime to inhibit growth of *Agrobacterium*. After 6 d leaf discs were transferred to SM-O plates with kanamycin at 100 mg l^{-1}. After 3 or 4 weeks, transgenic shoots appeared. These were placed in pots with MS-O agar containing 2 mg l^{-1} benzyladeninepurine, 0.2 mg l^{-1} naphthaleneacetic acid and 100 mg l^{-1} kanamycine for rooting. After roots had formed, three of four months after infection, the plantlets were transferred to the greenhouse and grown to maturity. Crosses were done with pollen from wild-type *N. glauca* plants.

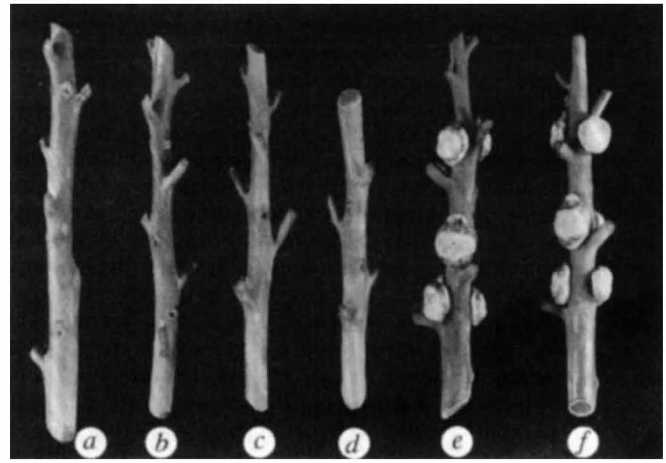

FIG. 4 Tumour induction on kanamycin-resistant *virF*-expressing transgenic *N. glauca* seedlings. No response after wounding (*a*), or infection with the avirulent helper strain LBA1115 (*b*), the *virB* mutant LBA1512 (ref. 6; *c*) or the *virE* mutant LBA1514 (ref. 6; *d*). Tumour formation after infection with the wild-type LBA1010 (ref. 23; *e*) or the *virF* mutant LBA1517 (ref. 6; *f*).

and protein may have a common evolutionary origin, especially considering that it is not naked DNA that is transported but a pilot protein (VirD2 in the case of T-DNA transfer) with a covalently attached DNA strand[1-3]. □

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1. Melchers, L. S. & Hooykaas, P. J. J. in *Virulence of Agrobacterium* (ed. Miflin, B. J.) 167–220 (Oxford University Press, Oxford, 1987).
2. Kado, C. I. *Crit. Rev. Plant Sci.* **10,** 1–32 (1991).
3. Zambryski, P. C. *A. Rev. Plant Physiol. Plant molec. Biol.* **43,** 465–490 (1992).
4. Melchers, L. S. *et al. Plant molec. Biol.* **14,** 249–259 (1990).
5. Otten, L. A. B. M. *et al. Molec. gen. Genet.* **199,** 189–193 (1985).
6. Hooykaas, P. J. J., Hofker, M., Den Dulk-Ras, H. & Schilperoort, R. A. *Plasmid* **11,** 195–205 (1984).
7. Otten, L. A. B. M. *et al. Molec. gen. Genet.* **195,** 159–163 (1984).
8. Vancanneyt, G., Schmidt, R., O'Conner-Sanchez, A., Willmitzer, L. & Rocha-Sosa, M. *Molec. gen. Genet.* **220,** 245–250 (1990).
9. Possot, O., d'Enfert, C., Reyss, I. & Pugsley, A. P. *Molec. Microbiol.* **6,** 95–105 (1992).
10. Dums, F., Dow, J. M. & Daniels, M. J. *Molec. genet. Genet.* **229,** 357–364 (1991).
11. Beijersbergen, A., Den Dulk-Ras, A., Schilperoort, R. A. & Hooykaas, P. J. J. *Science* **256,** 1324–1327 (1992).
12. Lessl, M., Balzer, D., Pansegrau, W., Lanka, E. *J. biol. Chem.* **267,** 20471–20480 (1992).
13. Goddijn, O. J. M. thesis, Leiden Univ. (1991).
14. Bevan, M. *Nucleic Acids Res.* **12,** 8711–8721 (1984).
15. Pietrzak, M., Shillito, R. D., Hohn, T. & Portykus, I. *Nucleic Acids Res.* **14,** 5857–5868 (1986).
16. Minton, N. P. *Gene* **31,** 269–273 (1984).
17. Chambers, S. P., Prior, S. E., Barstow, D. A. & Minton, N. P. *Gene* **68,** 139–149 (1988).
18. Ooms, G. *et al. Plasmid* **7,** 15–29 (1982).
19. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* 2nd edn (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).
20. Mattanovich, D. *et al. Nucleic Acids Res.* **17,** 6747 (1989).
21. Laemmli, U. K. *Nature* **227,** 680–685 (1970).
22. Burnette, W. M. *Analyt. Biochem.* **112,** 195–203 (1981).
23. Koekman, B. P., Hooykaas, P. J. J. & Schilperoort, R. A. *Plasmid* **7,** 119–132 (1982).
24. Hooykaas, P. J. J., Den Dulk-Ras, H., Ooms, G. & Schilperoort, R. A. *J. Bact.* **143,** 1295–1306 (1980).
25. Horsch, R. B. *et al. Science* **227,** 1229–1231 (1985).

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Co-existence of cationic and chloride components in odorant-induced current of vertebrate olfactory receptor cells

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ODORANT stimulation leads to a depolarization of olfactory receptor neurons[1–3]. A mechanism underlying this transduction, which occurs in the sensory cilia[3–6], involves a G-protein-mediated increase in adenylyl cyclase activity[7–10], and therefore a rise in internal cyclic AMP and consequent opening of a cAMP-gated cation channel on the plasma membrane[11–22]. Another mechanism, not as well established, involves the opening of an inositol trisphosphate-activated cation channel on the plasma membrane[23] as a result of phospholipase C activity[24,25]. In both cases, an influx of cations is thought to generate the depolarizing receptor potential. We now report, however, that the mechanism is actually more complex. The odorant-induced current appears to contain an inward chloride component also, which is triggered by calcium influx through the cation-selective channel. This newly found chloride component can be as large as the cationic component. The co-existence of cationic and chloride components in the odorant response, possibly unique among sensory transduction mechanisms, may serve to reduce variations in the transduction current resulting from changes in external ionic concentrations around the olfactory cilia. Our finding can explain the long-standing puzzle of why removal of most mucosal cations still does not diminish the amplitude of the olfactory receptor cell response[26–28].

Whole-cell recordings under voltage-clamp were made from single, dissociated olfactory receptor neurons of the newt (*Cynops pyrrhogaster*) while brief pulses of odorant (a mixture of *n*-amylacetate, isoamylacetate and limonene) were applied through a puffer pipette (for methods, see Fig. 1 legend). Similar experiments on adult tiger salamander (*Ambystoma tigrinum*) olfactory neurons gave identical results.

The involvement of a Cl^- conductance in olfactory transduction was first suggested by the effect of external Na^+ concentration, $[Na^+]_o$, on the reversal potential of the odorant-induced current. In physiological saline, odorant stimulation elicits current responses that are monophasic at all voltages and have a reversal potential near +5 mV (Fig. 1*a*). In the absence of external Ca^{2+}, this reversal potential depends strongly on external Na^+ (ref. 3); it shifts negatively by ~57 mV for a 10-fold reduction in $[Na^+]_o$ (Fig. 1*c*, solid line), consistent with the opening of a cation-selective conductance and the near-bicationic condition of such an experiment (external Na^+ and internal Cs^+, with isotonic Cl^- on both sides of the membrane; Fig. 1 legend). In the presence of external Ca^{2+}, however, the reversal potential became quite insensitive to $[Na^+]_o$ and remained near 0 mV even with very low external Na^+ (Fig. 1*b*, and dashed line in Fig. 1*c*), suggesting that there is another major current component in the olfactory response. This component cannot be carried by Ca^{2+}, because Ca^{2+} is not a major current carrier through the odorant-induced cation conductance[3,29]. It must be triggered instead by a rise in intracellular Cs^{2+}, most probably a Ca^{2+}-activated Cl^- current; a Ca^{2+}-activated K^+ current would have been blocked by the internal Cs^+ used in these experiments, or otherwise would have given a very negative reversal potential from 0 mV. The existence of a Ca^{2+}-activated Cl^- conductance has very recently been reported in frog olfactory cilia[30].

To check the involvement of chloride, we reduced the external Cl^- concentration, $[Cl^-]_o$, to 34 mM by replacement with gluconate. In this case, the odorant-induced current became conspicuously biphasic at +24 mV, consisting of an outward component followed by an inward component (Fig. 2*a*). The current–voltage relation measured at two different time instants during the response is shown in Fig. 2*b*. The positive shift of the reversal potential at later time (t_2) is consistent with a delayed Cl^- current subject to an equilibrium potential which is very positive from zero owing to the low $[Cl^-]_o$. All cells tested ($n = 10$) showed similar results, suggesting that the Cl^- current is probably present in all receptor cells.

The presence of a chloride component in the olfactory response was further confirmed using the chloride-channel blocker SITS, which selectively removed the inward current in the biphasic odorant response elicited in low external Cl^- and at a positive membrane potential, as already described (Fig. 3*a*). The effect of SITS was rapid and reversible. This drug had little effect on the odorant response in the absence of external Ca^{2+}, indicating that it blocked the Cl^- component specifically (data not shown). The time courses of the cationic (trace a) and Cl^- (trace b; obtained by subtraction, see legend) currents from the experiment shown in Fig. 3*a* are plotted in Fig. 3*b*. The Cl^- current was more prolonged; it was also activated more slowly than the cationic current, consistent with its induction by a Ca^{2+} influx through the cation-selective channel. Figure 3*c* shows a similar experiment, except in this case the cell was bathed in physiological saline and the membrane potential was held at −54 mV, close to the physiological resting potential of these cells. 2 mM SITS again reduced the amplitude of the odorant-induced inward current, with the separated cationic (trace a) and Cl^- (trace b) components being shown in Fig. 3*d*. From a

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