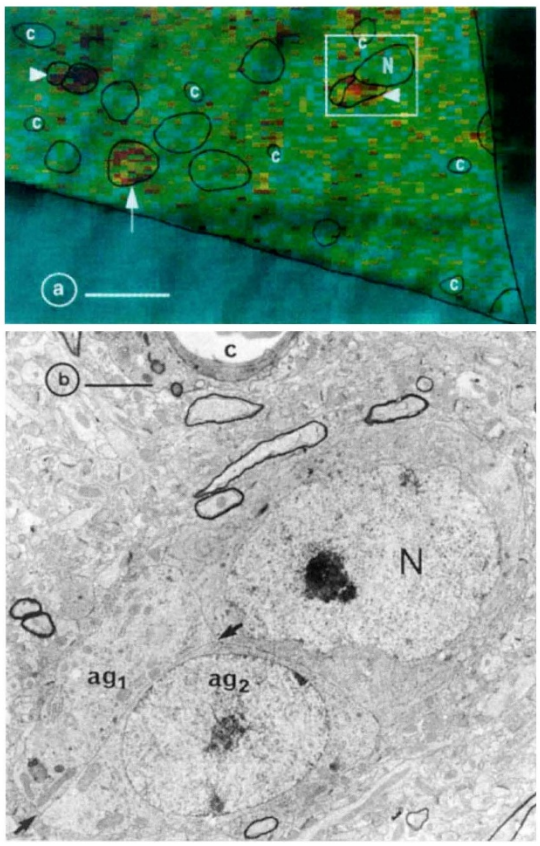

FIG. 3 Identification of [^{14}C]GABA-accumulating cells in layer 4 of the visual cortex of rats using SIAMS (a) correlated with transmission electron microscopy (b) in serial sections (a, 100 nm thick; b, 70 nm thick). a, The corner of an ultrathin section contains three areas of high ^{14}C content of about the same size. Electron microscopic examination revealed that one of them (vertical arrow) corresponds to a non-pyramidal neuron, whereas the other two represent two adjoining ^{14}C-labelled glial cells each (triangles). Black lines outline the edge of the section, capillaries (c), labelled and unlabelled neurons and labelled glial cells as identified in consecutive light and electron microscopic sections. b, The framed area in *a* is shown in the consecutive electron microscopic section where a capillary, an unlabelled neuron (N) and the two astroglial cells (ag_1,ag_2) can be identified. The boundary between the glial cells is indicated (arrows). Scale bars: a, 20 μm; b, 2 μm.

used 2-deoxy[^{14}C]glucose technique[16]. SIAMS is applicable to electron microscopic sections of about 80–100 nm thick, which further increases the resolution of the localization as well as the identification of the cellular components. It will be important to increase both the resolution and the sensitivity further for the quantitative localization of ligand binding sites by neurotransmitter receptors and ion channels which can only be localized at present with low resolution. The prospect of using ligands labelled with different isotopes shows how SIAMS imaging can potentially reveal the molecular machinery of chemical communication in the brain. □

Methods

Two adult Wistar rats were deeply anaesthetized with sodium pentobarbital (Sagatal; 45 mg kg^{-1}, i.p., supplemented as required) and mounted in a stereotaxic apparatus. Following a small craniotomy, the occipital cortex was injected unilaterally through glass micropipettes (tip, 30–50 μm) at two sites, perpendicular to the superior sagittal sinus and at 40 degrees to the vertical plane. Radiolabelled GABA (209 mCi mmol^{-1}, Amersham) was dissolved in artificial cerebrospinal fluid at a concentration of 0.95 mM, diluted further by the injection into the brain. Along each track (4–5 mm tangentially, latero-ventral from the pia), 5–8 injections were placed 0.5 mm apart delivering a total of 0.75 μl GABA (~700 nmol), slowly over 15 min. Animals received an overdose of anaesthetic 50 min after the start of the first injection, followed by transcardial perfusion with a fixative containing 2.5% glutaraldehyde, 0.5% paraformaldehyde in 0.1 M phosphate buffer. The brains were sectioned sagittally (cross sectioning to the tracks), the sections (60-μm-thick) were treated with osmium tetroxide (0.1%) to reduce shrinkage, and embedded in epoxy resin (Durcupan, Fluka)[10,11]. Strips of cortex (0.2–0.3 mm wide) from pia to white matter at different lateral distances from the injection tracks were sectioned further at 0.5 μm thickness on an ultramicrotome. Serial sections were alternately mounted either on Al stubs for SIAMS, or on glass slides for either the immunocytochemical visualization of GABA[17] using polyclonal antibodies (diluted 1:6,000)[18] and the indirect immunoperoxidase method, or for toluidine-blue-staining to reveal cellular elements in the light microscope. The sections for electron microscopy were picked up on pioloform-coated single-slot grids, and contrasted with uranyl acetate and lead citrate.

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The octadecanoid signalling pathway in plants mediates a response to ultraviolet radiation

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MANY plant genes that respond to environmental and developmental changes are regulated by jasmonic acid, which is derived from linolenic acid via the octadecanoid pathway[1]. Linolenic acid is an important fatty-acid constituent of membranes in most plant species and its intracellular levels increase in response to certain signals. Here we report that irradiation of tomato leaves with ultraviolet light induces the expression of several plant defensive genes that are normally activated through the octadecanoid pathway after wounding[2]. The response to ultraviolet light is blocked by an inhibitor of the octadecanoid pathway and it does not occur in a tomato mutant defective in this pathway. The ultraviolet irradiation maximally induces the defence genes at levels where cyclobutane pyrimidine dimer formation, an indicator

of DNA damage, is less than 0.2 dimers per gene. Our evidence indicates that this plant defence response to certain wavelengths of ultraviolet radiation requires the activation of the octadecanoid defence signalling pathway.

The response to ultraviolet (UV) light has been well studied in bacteria[3] and yeast[4] and in animal cell lines at 254 nm wavelength within the UV-C range (below 280 nm), which activates a set of genes that are also induced by UV-A (above 315 nm) and UV-B (280–315 nm) irradiation[5]. UV-C also induces the same photoproducts induced in DNA by UV-B[6] but at higher frequency[7]. To compare the response to ultraviolet irradiation in plants with studies of animals, the induction of two defensive proteinase-inhibitor genes, normally induced in leaves in response to herbivory, was analysed in wounded and unwounded tomato leaves that were exposed to both UV-C and UV-B irradiation.

After exposing the leaves to increasing doses of 254-nm (peak wavelength) ultraviolet irradiation, total RNA was isolated and analysed for proteinase inhibitor I and II messenger RNAs (Fig. 1*a*). Inhibitor I and II genes were both induced by UV-C in a similar dose-dependent manner, and the highest levels of mRNA were observed after irradiation in the range of 2 kJ m^{-2}. The levels of the constitutively expressed ubiquitin gene did not change in response to UV irradiation or wounding. Moreover, the synthesis of proteinase inhibitors responded incrementally to increases in UV exposure from 0.5 to 2 kJ m^{-2} (Fig. 1*b*), but at higher doses the effects began to diminish. UV irradiated wounded plants accumulated roughly 50% more inhibitor-I protein than UV irradiated unwounded plants (Fig. 1*b*), indicating that the UV induction and wound induction were partially additive. Repeated wounding has previously been shown to have an additive effect[8], therefore UV treatment seems to mimic wounding. Additionally, the time course of the proteinase inhibitor I mRNA induction in response to UV irradiation is similar to that during wound induction (Fig. 1*c*).

We next explored whether the response of the two proteinase inhibitor genes to UV might be mediated through the octadecanoid pathway, through which several defensive genes (including the inhibitor genes) are regulated in response to wounding[2,9]. Plants were treated with salicylic acid, a potent inhibitor of the pathway[10], just before UV-C irradiation. Both wound induction and UV induction of the inhibitor I and II proteins were abolished by the presence of sailicylic acid (Fig. 2*a*). A mutant tomato line (JL-5) was recently identified, which carries a mutation in the octadecanoid pathway that blocks the wound-induction of defensive genes[11]. The UV induction of proteinase inhibitor I mRNA is also blocked in the JL-5 mutant line (Fig. 2*b*). These findings are consistent and indicate that the UV response of the proteinase inhibitor I and II genes might be mediated by the same intracellular defence-signalling pathway involved in wounding. Several systemic wound-response genes that are activated through the octadecanoid pathway[2,9] are also UV-C inducible (Table 1). A set of pathogenesis-related proteins (PR1a, PR3a and PR3), which accumulate in response to viral and microbial pathogens and that are not wound inducible[12], are not induced by UV-irradiation in tomato plants. However, a PR1 gene in tobacco plants was recently reported to be induced several hours after UV irradiation[13,14]. Although the mechanism for this latter response is not known, the UV induction of the pathogenesis-related proteins was attributed to active oxygen species, which have been implicated in the pathogen induction of pathogenesis-related genes in tobacco plants[14] but not in the induction of proteinase-inhibitor genes or other wound-inducible genes.

The UV-induced DNA damage is proposed to be the initial step in the activation of a particular class of genes in animal systems, and has been termed the UV response[15]. We looked for an analogous response in plants by measuring UV-C induction of cyclobutane pyrimidine dimer (CPD) formation in the inhibitor I and II genes. The results (Fig. 3) show that an average of about 1 CPD was introduced into the *Eco*RI DNA fragment (~4.2 kilobases) containing the inhibitor I gene (~1.3 kb), when plants were irradiated at 8 kJ m^{-2}. At UV doses that induce maximal proteinase inhibitor I mRNA and protein accumulation (1–2 kJ m^{-2}), roughly 0.2 CPDs were found in the same DNA fragment, and an even lower level is probably introduced into the inhibitor I gene (~30% of the *Eco*RI fragment length). Although the DNA sequences of the proteinase inhibitor I and II genes are unrelated, the two genes are coordinately induced by wounding[8] and ultraviolet light (Fig. 1). As CPD formation depends on the DNA sequence[6,16], we directly compared the UV-induced CPD levels in the inhibitor I DNA fragment (~4.2 kb) with those in the *Hin*dIII DNA fragment (~5.0 kb) containing the inhibitor II gene (~1 kb). The formation of CPDs in both inhibitor fragments was found to be similar in wounded and unwounded plants (Fig. 3), that is, the maximum expression in both genes occurs at less than ~0.2 CPD per proteinase inhibitor gene. Moreover, CPDs were formed in UV-irradiated JL-5 mutant tomato plants at levels comparable to wild type plants (Fig. 3). It is possible that the yield of other UV photoproducts (for example 6-4-pyrimidine pyrimidones)

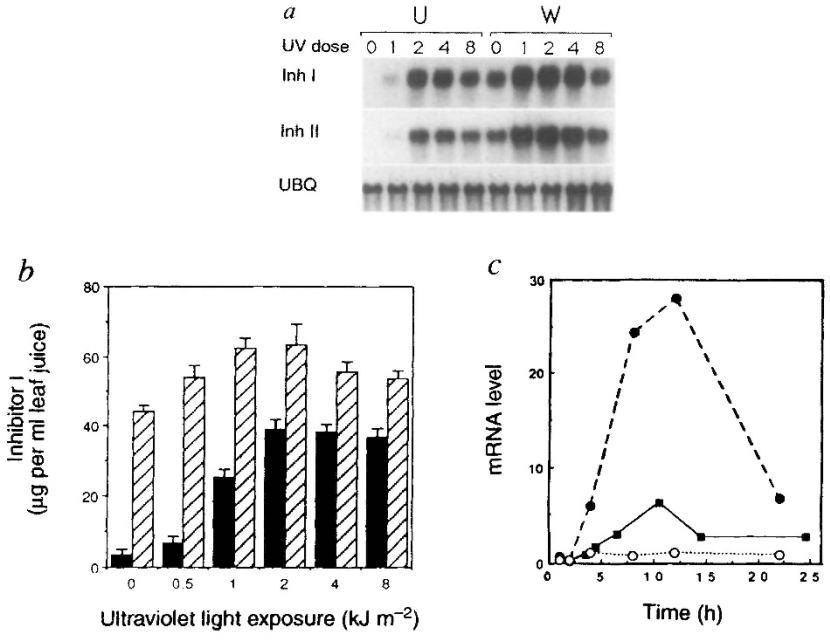

FIG. 1 UV dose–response of the proteinase inhibitor I and II in leaves of 14-day-old tomato plants. *a*, Plants were wounded once with a haemostat and UV-irradiated 2.5 h after wounding. Total RNA was isolated from the whole leaves 6.5 h after UV irradiation (9 h after wounding) and analysed for the proteinase inhibitor I (Inh I)[28], the proteinase inhibitor II (Inh II)[29] or ubiquitin (UBQ) RNA. The lanes correspond to RNA isolated from unwounded (U) or wounded (W) leaves UV-irradiated at a dose of 1, 2, 4 or 8 kJ m^{-2}. Lanes 0, RNA isolated from control plants. *b*, Unwounded (solid bars) and wounded (hatched bars) tomato plants were UV-irradiated with increasing doses, incubated under standard conditions for 25 h and analysed for proteinase inhibitor I protein content by immunoradial diffusion. Error bars, s.e.m. of six different experiments. *c*, Densitometric quantification of the inhibitor I northern blots. Filled squares, wounding; filled circles, irradiation at 2 kJ m^{-2}; open circles, unwounded, non-irradiated control plants.

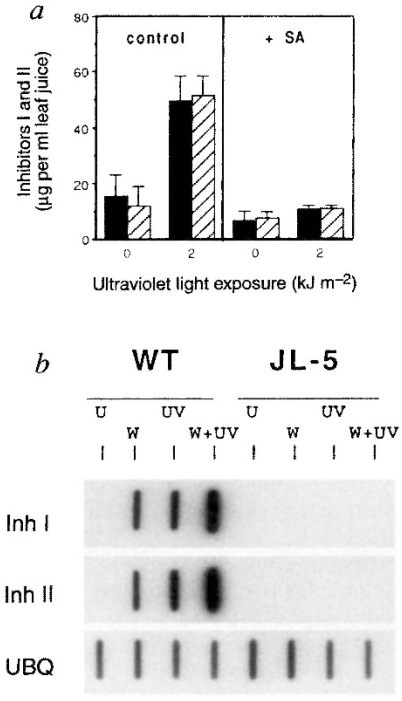

FIG. 2 Inhibition in leaves of 14-day-old tomato plants of wound- and UV-inducible proteinase inhibitor I and II synthesis by salicylic acid and by a mutation in the octadecanoid pathway. *a*, Excised tomato plants were supplied with 1 mM Salicylic acid (+SA) or water (Control) through the cut stems, 1 h before UV irradiation (2 kJ m^{-2}), incubated together with the non-irradiated control plants (0 kJ m^{-2}) for 25 h under standard conditions and analysed for proteinase inhibitor I (solid bars) and II (hatched bars) contents. Error bars, s.e.m. of two different experiments (6 plants each). *b*, RNA was isolated from UV-irradiated (2 kJ m^{-2}) wild-type (WT) and mutant (JL-5) plants. (U) unwounded, (W) wounded, (UV) UV-irradiated and (W + UV) wounded and UV-irradiated leaves. (Inh I) inhibitor I, (Inh II) inhibitor II and (UBQ) ubiquitin mRNAs.

might have been found to correlate differently with the induction response, but these lesions are present at even lower frequencies than CPD in most DNA[17]. The relative low concentration of CPD found in the AT-rich (~70%) sequences of the proteinase inhibitor I and II genes, after exposure to very high UV dose, may be explained by the presence in plants of shielding mechanisms (cuticular waxes, trichomes, pigments and flavonoids) that efficiently absorb or scatter ultraviolet light[18].

The proteinase inhibitor genes that are activated in tomato

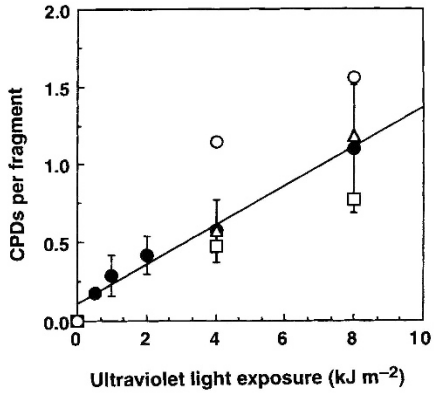

FIG. 3 UV-induced cyclobutane pyrimidine dimer (CPD) formation in genomic DNA fragments containing the proteinase inhibitor I and II genes. *Eco*RI or *Hin*dIII digested leaf DNA was incubated with T4 endo V nuclease, separated by alkaline agarose gel and probed for the proteinase inhibitor I or II genes. The data represent the average yield of CPDs per 4.2-kb DNA fragment and s.d. for three different experiments obtained for the proteinase inhibitor I DNA fragment in unwounded leaves (filled circles). Average of two different experiments obtained for the unwounded proteinase inhibitor II DNA fragment (triangles), the wound-induced proteinase inhibitor II DNA fragment (squares) and the inhibitor I DNA fragment of JL-5 tomato mutants (open circles).

TABLE 1 UV-C induction of systemic wound-response genes in leaves of tomato plants

	WT		JL-5	
Gene product	Wounded	UV irradiated	Wounded	UV irradiated
Proteinase inhibitor I	+	+	–	–
Proteinase inhibitor II	+	+	–	–
SH proteinase inhibitor	+	+	–	–
Asp proteinase inhibitor	+	+	–	–
Polyphenol oxidase	+	+	–	–
Leucine aminopeptidase	–	+	–	–
Threonine deaminase	+	+	–	–
PR1a	–	–	–	–
PR3a	–	–	–	–
PR3	–	–	–	–
Ubiquitin	(–)	(–)	(–)	(–)

After wounding or UV irradiation of wild-type (WT) and mutant (JL-5) plants, RNA was isolated (7.5 h after UV irradiation). Northern blots were probed with: proteinase inhibitor I and II complementary DNAs[29], SH proteinase inhibitor[30], Asp proteinase inhibitor[30], polyphenol oxidase (from P. Constabel), leucine aminopeptidase (from L. Walling), threonine deaminase[30]; tobacco acidic PR1a, PR3a and basic PR3 cDNAs (from J. Bol) and *Antirrhinum* ubiquitin (from C. Martin). +, gene induction; –, no gene induction or RNA present at low levels; (–), no gene induction but RNA constitutively present.

leaves by UV-C irradiation are also activated by irradiation with the more ecologically relevant 310 nm UV-B light (Table 2) but are not induced upon UV-A irradiation (data not shown). Although UV-B was less effective than either wounding or UV-C in inducing the synthesis of the proteinase inhibitors I and II, UV-B activation was about three times higher than that observed in leaves of untreated control plants. No activation occurred in leaves of the JL-5 tomato mutant that has an impaired octadecanoid signalling pathway. This indicates that the octadecanoid pathway in tomato plants probably mediates both UV-C and UV-B responses of the proteinase inhibitor I and II genes.

In animal cells, free-radical formation has been shown to occur in response to UV-A and UV-B irradiation[19], potentially leading to membrane perturbation, activation of phospholipases and the release of arachidonic acid[20–22]. Arachidonic acid, the precursor of the prostaglandins in animals, has been shown to be released on UV-C irradiation of cell membranes[23]. In plants, which do not contain arachidonic acid or prostaglandins, linolenic acid is released from membranes in response to wounding, and is converted to the prostaglandin analogues phytodienoic acid and jasmonic acid via the octadecanoid pathway[2]. These prostaglandin analogues are powerful inducers of the systemic wound-response

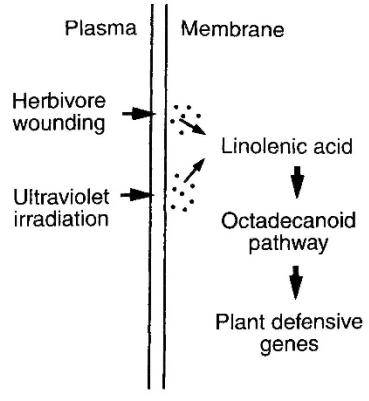

FIG. 4 Proposed model for the signalling of the ultraviolet response in tomato leaves.

TABLE 2 Extent of induction of proteinase inhibitors in leaves

Inducer	Proteinase inhibitor I		Proteinase inhibitor II	
	WT	JL-5	WT	JL-5
Control	4.9 ± 1.2	0	3.2 ± 1.3	0
Wounding	19.7 ± 1.0	0	21.5 ± 1.9	0
UV-C	20.0 ± 1.0	0	20.9 ± 2.1	0
UV-B	13.6 ± 1.0	0	10.9 ± 1.3	0

Comparison of the induction of proteinase inhibitors I and II in leaves of 11-day-old wild-type (WT) and mutant (JL-5) tomato plants by wounding, UV-B and UV-C irradiation. In parallel, under the same light conditions, tomato plants were either wounded, or irradiated with 254 nm UV-C (2 kJ m^{-2}) or with 310 nm UV-B (30 to 40 kJ m^{-2}). The control and the treated plants were incubated under standard conditions for 25 h and the content of the proteinase inhibitor I and II proteins was analysed by immunoradial diffusion. Mean ± s.e.m. of three different experiments.

genes. To explain the similarities between wounding and the UV response in activating systemic wound-response genes, we suggest that UV irradiation results in the perturbation of plant membranes and/or the activation of lipases to cause the release of linolenic acid, which engages the intracellular octadecanoid signal-transduction pathway to activate the wound inducible genes (Fig. 4). It is also possible that DNA damage might be the initial signal that activates the octadecanoid pathway even though only low levels of DNA damage occurred in tomato leaves upon UV-C irradiation. However, this damage could result in the production of a signal that is released from the nucleus into the cytoplasm which then activates a membrane-bound signalling process, as proposed in animal cell lines[24].

A long-term ozone depletion over the mid-latitudes of both the northern and southern hemispheres has resulted in elevated UV exposures over extended periods. Increased UV exposures were reported to alter the competitiveness of some plant species and possibly to alter plant communities and ecosystems over a relatively short evolutionary period[25]. It is possible that the activation of signalling pathways for defence genes by increased levels of ultraviolet light may contribute to these changes. □

Methods

Tomato plants (*Lycopersicon esculentum* (L.) Mill., cv Castelmart) were grown from seeds under standard conditions (17 h of light (250 μE m^{-1} s^{-1}) at 27 °C and 7 h in the dark at 18 °C. We used leaves and cotyledons of 10-day-old plants (two small developed leaves) and irradiation was done with four low-pressure mercury lamps (predominantly 254 nm) at a flux of ~15 W m^{-2} (measured with a Spectronics DM-254 N UV meter). The UV-C treated plants did not show any phenotypic evidence of stress, except at UV doses of 8 kJ m^{-2}, where shiny spots appeared on the leaves. Also, irradiated plants showed a slower growth rate than control plants. The UV-B experiments were done with two UV-B lamps (peak wavelength, 310 nm) and the flux (~35 W m^{-2}) was measured with a UVP UV meter.

Wounded and/or UV-irradiated plants were further incubated in the light under standard conditions for different periods of time, and the induction of the proteinase inhibitor I and II genes was measured by northern blots analysis. Alternatively, the proteinase inhibitor I and II protein content was analysed by immunoradial diffusion as previously described[8]. For CPD measurements after UV-C irradiation, unwounded or wounded tomato leaves were collected in the dark and frozen in liquid nitrogen to limit DNA repair by endogenous enzymes. Total leaf DNA was first digested with *Eco*RI or *Hin*dIII and then incubated with T4 endo V nuclease (which nicks DNA strands at CPD sites) or mock treated[26]. The average number of CPDs per fragment was determined from the fraction free of CPDs using the Poisson equation ($-\ln(P_0)$) (ref. 27).

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Insertional mutagenesis and rapid cloning of essential genes in zebrafish

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LARGE-SCALE chemical mutagenesis screens in zebrafish have led to the isolation of thousands of lethal mutations in genes that are essential for embryonic development[1,2]. However, the cloning of these mutated genes is difficult at present as it requires positional cloning methods. In *Drosophila*, chemical mutagenesis screens were complemented with P-element insertional mutagenesis which facilitated the cloning of many genes that had been identified by chemical lesions[3,4]. To facilitate the cloning of vertebrate genes that are important during embryogenesis, we have developed an insertional mutagenesis strategy in zebrafish using a retroviral vector. Here, in a pilot screen of 217 proviral insertions, we obtained three insertional mutants with embryonic lethal phenotypes, and identified two of the disrupted genes. One of these, *no arches*, is essential for normal pharyngeal arch development, and is homologous to the recently characterized *Drosophila* zinc-finger gene, *clipper*, which encodes a novel type of ribonuclease[5]. As it is easy to generate tens to hundreds of thousands of proviral transgenes in zebrafish[6], it should now be possible to use this screening method to mutate and then rapidly clone a large number of genes affecting vertebrate developmental and cellular processes.

The infection of blastula-stage zebrafish embryos with murine leukaemia virus/vesicular stomatitis virus pseudotyped retroviral vectors[7,8] results in the germline transmission of proviral insertions[6,9]. Integration of proviral DNA into the mouse genome can disrupt essential genes[10]. In an effort to isolate insertional mutations in zebrafish, we generated and inbred 217 proviral transgenes and screened for mutant phenotypes that were linked to those transgenes. The virus used to generate the insertions