

preincubated with iberiotoxin, paxilline, 4-AP or NS1619 for 3 min in a rapidly stirred oxygenated chamber at 37 °C, then mixed with 10⁸ IgG-opsonized microbes. Phagocytosis was measured as described⁷ using fluorescent rather than radiolabelled bacteria.

Superoxide dismutase activity

H₂O₂ production was measured in a xanthine-xanthine-oxidase O₂⁻-generating system by the bleaching of phenol red³⁰.

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OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*

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Active oxygen species (AOS) generated in response to stimuli and during development can function as signalling molecules in eukaryotes, leading to specific downstream responses^{1,2}. In plants these include such diverse processes as coping with stress (for example pathogen attack³, wounding⁴ and oxygen deprivation⁵), abscisic-acid-induced guard-cell closure⁶, and cellular development (for example root hair growth⁷). Despite the importance of signalling via AOS in eukaryotes, little is known about the protein components operating downstream of AOS that mediate any of these processes. Here we show that expression of an *Arabidopsis thaliana* gene (*OXI1*) encoding a serine/threonine kinase is induced in response to a wide range of H₂O₂-generating stimuli. *OXI1* kinase activity is itself also induced by H₂O₂ *in vivo*. *OXI1* is required for full activation of the mitogen-activated protein kinases (MAPKs) MPK3 and MPK6 after treatment with AOS or elicitor and is necessary for at least two very different AOS-mediated processes: basal resistance to *Peronospora parasitica* infection, and root hair growth. Thus, *OXI1* is an essential part of the signal transduction pathway linking oxidative burst signals to diverse downstream responses.

Our attempts at identifying signalling components acting downstream of AOS signals focused on protein kinases as likely candidates. To identify the kinase genes specifically involved in AOS signalling in *Arabidopsis*, we compared kinase-encoding transcript levels in H₂O₂-treated (to mimic an oxidative burst) and control *Arabidopsis* seedlings by differential display of amplified complementary DNA fragments. In this screen, kinase-encoding cDNA sequences were amplified by polymerase chain reaction (PCR) with degenerate primers complementary to sequences conserved between protein kinases (data not shown). One gene identified in this way contained a coding region corresponding to a putative serine/threonine kinase (At3g25250) and was named *OXI1*, for *OXIDATIVE SIGNAL-INDUCIBLE1*.

OXI1 induction in response to AOS (H₂O₂) was confirmed by northern blot analysis (Fig. 1a). H₂O₂-treated seedlings were viable (data not shown), confirming previous reports that this stimulus was not lethal^{6,8}. As described above, AOS signalling in plants is triggered by a range of environmental stresses^{3–6}. We therefore examined the expression pattern of *OXI1* in response to a variety of stress treatments. *OXI1* was expressed to relatively high levels, particularly after wounding and treatment with cellulase (Fig. 1b). The prolonged kinetics of *OXI1* expression seen in response to H₂O₂ and cellulase contrasted with the rapid transient seen in response to wounding. These differences are probably due to the constant presence of the H₂O₂ and cellulase signals in comparison with the

discrete nature of the wounding signal, and the smaller number of cells affected in the latter case. Spatial patterns of transcription of the *OX11* promoter in response to these two treatments were measured with an *OX11::GUS* reporter gene fusion construct (Fig. 1c). GUS (β -glucuronidase) staining of the *OX11::GUS* fusion lines after treatment with cellulase showed that the promoter was activated across the entire surface of the cotyledons (Fig. 1c, middle panel) that was consistent with the uniform degradation of cellulose by this enzyme. Conversely, wounding with a sharp razor blade increased GUS levels only along the cut edge of the wounded tissue and along the severed vascular tissues (Fig. 1c, right panel). These specific regions have been shown to produce H_2O_2 in response to wounding in plants⁴ including *Arabidopsis* (Supplementary Fig. 1; ref. 9). Control (untreated) seedlings did not show any staining (Fig. 1c, left panel) in cotyledons or leaves. We also found that *OX11::GUS* fusion lines exhibited GUS expression along growing fungal hyphae of the virulent *P. parasitica* isolates Maks9 and Emco5, particularly in the adjacent layer of two to four cells and in cells penetrated by fungal haustoria (Fig. 2a). The staining pattern indicates that AOS production might be limited to cells in close proximity to the invading fungus. This is consistent with AOS production during pathogenesis^{10,11}, in which AOS generation in cells adjacent to virulent fungi has been shown¹².

In addition to stress-induced expression of *OX11* in the aerial parts of the plant, the *OX11* promoter is always active in the root. Here it is more highly expressed under growth conditions imposing a mild stress on the root, for example when seedlings are grown on ordinary agar instead of refined agar (Phytigel) (Fig. 3a; Supplementary Fig. 2), and often to a particularly high level in root hair cells. This would be consistent with the necessity of an AOS signal for normal root hair cell development⁷. Taken together, these data indicate that *OX11* expression might be mediated by AOS produced in plant cells in response to stress and during root hair development, and they point to a role for the OX11 protein in responses involving AOS.

To determine the specific function of OX11 in AOS-mediated responses, we isolated an *oxi1*-null mutant line from the Wisconsin T-DNA insertion populations¹³ by using PCR-based screening (data not shown). The *oxi1* mutant expresses only a transcript encoding a truncated protein (the first 23 residues) lacking the catalytic site (data not shown), which is therefore non-functional. Complemen-

tation of the *oxi1* mutant with the wild-type *OX11* gene confirmed that the T-DNA insertion created a null mutation and that this gene was responsible for all the phenotypes described below.

The necessity of OX11 for resistance to a virulent pathogen was confirmed by comparing the susceptibility of the *oxi1* mutant and the wild type to pathogen infection. Assessment of the extent of fungal sporulation showed that *oxi1* seedlings have a significantly enhanced susceptibility to the virulent *P. parasitica* isolate Emco5 in comparison with the Ws-2 wild type (Fig. 2b and c). Resistance to the avirulent isolate Emoy2 was not removed in the *oxi1* mutant (data not shown). Because AOS are also likely to be produced in response to avirulent fungi, this implies that OX11 either is not involved in host-specific resistance or is a redundant component in this context.

Cessation of root hair growth is a characteristic of some *rhd2* mutants¹⁴ that fail to generate necessary AOS during root hair development⁷. We therefore examined the *oxi1* mutant for altered root hair growth. The average root hair length of the *oxi1*-null mutant is significantly less than that of the Ws-2 wild type (226.2 and 300.3 μ m, respectively). There was also a greater proportion of very short root hairs in *oxi1* than in the wild type; this effect was more marked for roots growing through air than for those in contact with the agar (Fig. 3b and 3c; data not shown).

To test directly whether OX11 kinase activity *in vivo* was induced by H_2O_2 , and hence to show that OX11 acts downstream of AOS, OX11 protein kinase activity was measured in *Arabidopsis* leaves (Fig. 4a). The addition of H_2O_2 resulted in strong activation of OX11 at 5 min of treatment. Cellulase treatment also induced OX11 activity in *Arabidopsis* leaves, although to a much higher level and over a longer period than in response to H_2O_2 . When leaves of *oxi1* mutant plants were analysed, OX11 protein could not be detected, and activation of OX11 by both H_2O_2 and cellulase was completely abrogated. These data show that OX11 is a protein kinase that is activated in response to H_2O_2 and cellulase.

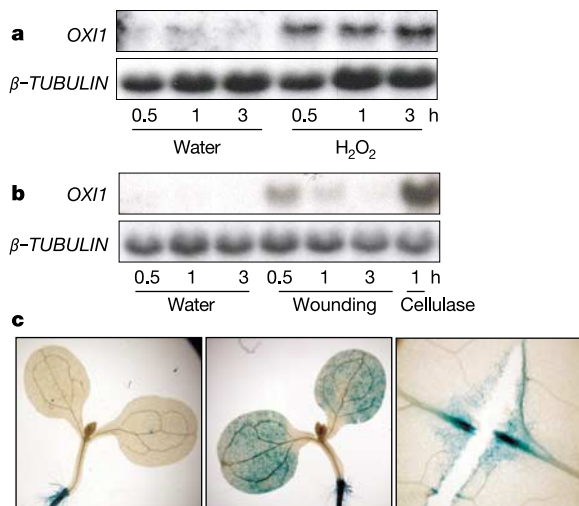


Figure 1 H_2O_2 , cellulase and wounding increase the expression of *OX11*. **a**, Induction of *OX11* in seedlings treated with H_2O_2 (10 mM) or water. **b**, Induction of *OX11* by cellulase (0.01% w/v) or wounding. *OX11* expression levels were determined by northern analysis. **c**, Seedlings containing an *OX11::GUS* gene construct were incubated in water (left panel) or 0.1% w/v cellulase solution (middle panel). In the right panel, cotyledons were cut across the surface with a sharp razor blade. After 3 h, tissues were stained for GUS expression²⁵.

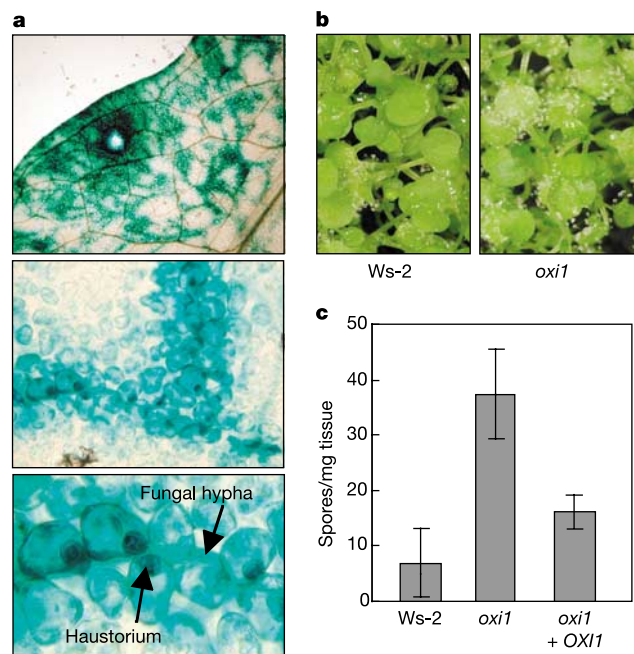


Figure 2 *OX11* is necessary for basal resistance to *P. parasitica*. **a**, Plants containing an *OX11::GUS* gene construct were infected with virulent *P. parasitica* (Maks9) and stained for GUS expression²⁵ 7 days after infection. Infected leaves show induction along fungal hyphae (top panel) and in cells containing fungal haustoria (middle panel, close-up in bottom panel). **b**, Infection levels of *oxi1* and Ws-2 wild-type seedlings 7 days after infection. **c**, Seedlings were infected with Emco5 and the level of fungal infection was assessed by determining the extent of sporulation on wild-type, *oxi1* mutant and *oxi1* complemented with wild-type *OX11* (*oxi1* + *OX11*) 6 days after infection (means \pm s.e.m., $n = 3$).

Activation of two of the *Arabidopsis* MAPKs—MPK3 and MPK6—is known to be essential for signal transduction in response to H₂O₂ (ref. 15) and elicitor treatment¹⁶. MPK6 is also activated on wounding¹⁷, and SIMK, the alfalfa homologue of MPK6, regulates root hair growth¹⁸. To gain insight into the molecular targets of OX11, H₂O₂- and cellulase-triggered activation of these kinases was investigated in the *oxi1* mutant. In the wild type, both MPK3 and MPK6 displayed increased activity after treatment with H₂O₂ and cellulase (Fig. 4b and c, respectively). However, in the *oxi1* mutant H₂O₂- and cellulase-triggered activation of both MPK3 and MPK6 was substantially reduced. Hence, OX11 is needed for the full activation of MPK3 and MPK6 in response to AOS, and the phenotypes of the *oxi1* mutant might be attributable to reduced signalling through these MAPKs. In agreement with this notion, overexpression of an OX11-YFP-c-Myc fusion protein in protoplasts increased H₂O₂-induced MPK3 activity (data not shown), supporting the role for OX11 in mediating the AOS-induced activation of the MAPKs. It is important to note that *OX11* gene expression and OX11 and MPK3/6 kinase activities were upregulated more strongly in response to cellulase than to H₂O₂. This might indicate that H₂O₂ treatment does not fully mimic a genuine oxidative burst generated by a stress signal such as cellulase and is therefore less effective. Timing, magnitude and spatial localization of the AOS signal in a real oxidative burst might be important in terms of activating downstream cellular processes. Alternatively, cellulase treatment might produce additional (to AOS) signals within the cell, for example by acting as an elicitor, and these other signals might also be capable of upregulating *OX11* gene expression and activity of the OX11, MPK3 and MPK6 kinases.

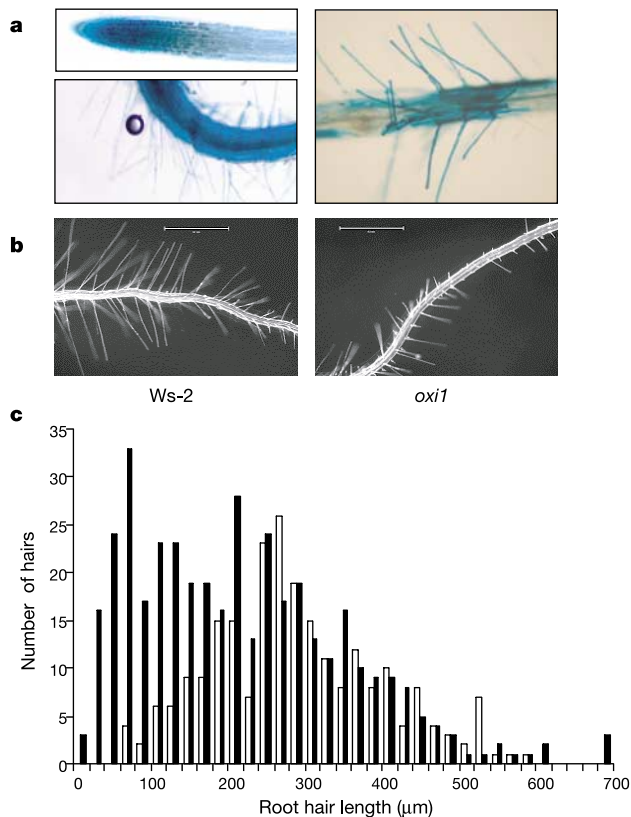


Figure 3 *OX11* is necessary for normal root hair development. **a**, Seedlings containing an *OX11::GUS* gene construct were grown on 0.8% agar and subsequently stained for GUS expression. GUS was always expressed in the root, often to particularly high levels in root hair cells. **b**, Visual comparison of *oxi1* and Ws-2 wild-type roots grown through air. **c**, Distribution of mature root hair lengths of *oxi1* seedlings (filled bars) and Ws-2 wild-type seedlings (open bars) with roots growing through air. For complementation data, see Supplementary Fig. 3.

The findings described above indicate that the AOS-inducible *OX11* gene, encoding a serine/threonine kinase, is transcriptionally upregulated in response to wounding and pathogen attack, stimuli that are associated with H₂O₂ generation^{3,4}. In addition, the *OX11* promoter is active in roots, particularly in root hairs under growth conditions imposing a mild stress. H₂O₂ is uncharged and relatively stable, properties that allow this molecule to cross membranes and reach neighbouring cells, as shown in tobacco epidermal peels¹⁹. H₂O₂ might therefore function as a local signalling molecule. Accordingly, wounding and infection with *P. parasitica* increased expression of the *OX11::GUS* reporter construct in several cell layers adjacent to the wounded or infected cells (Figs 1c and 2a, respectively). The OX11 protein is essential for basal resistance against a virulent pathogen, because the *oxi1*-null mutant showed an enhanced susceptibility phenotype with an elevated production of sporangiophores (Fig. 2b, c). OX11 is also necessary for normal root hair development during stress (Fig. 3b, c), root hair development itself requiring AOS generated by the *Arabidopsis* NADPH oxidase AtrbohC/RHD2 (ref. 7). Notably, *OX11* falls into the same kinase gene family as the *INCOMPLETE ROOT HAIR ELONGATION (IRE)* gene, which is also involved in root hair growth²⁰. The OX11 protein has protein kinase activity that is induced *in vivo* by H₂O₂

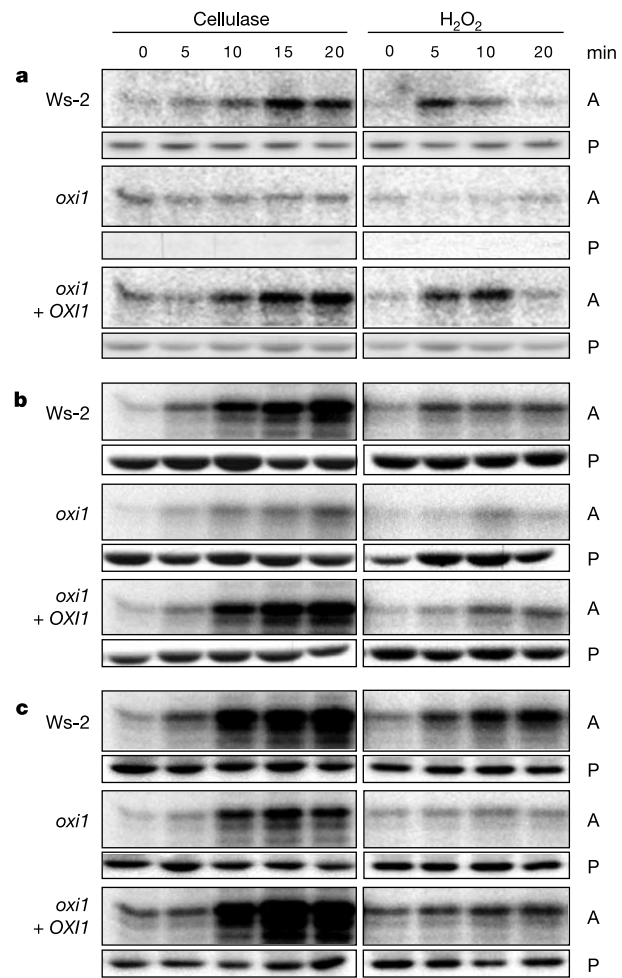


Figure 4 OX11 kinase activity is induced by H₂O₂ and cellulase, and *OX11* is necessary for MPK3 and MPK6 activation. **a**, Cellulase and H₂O₂ induce activity of the native OX11 kinase, immunoprecipitated with an anti-OX11 antibody from *Arabidopsis* leaves. **b, c**, MPK3 (**b**) and MPK6 (**c**) activity in cell extracts, assessed by immunocomplex kinase assays. Samples were treated with H₂O₂ (10 mM) or cellulase (0.1% w/v). Myelin basic protein was used as a substrate. A, activity; P, protein levels, determined by western blotting with antibodies specific for OX11 (**a**), MPK3 (**b**) and MPK6 (**c**).

and cellulase (Fig. 4a). Downstream (indirect) targets of OX11 are likely to include MPK3 and MPK6, because *OX11* is required for full activation of these kinases (Fig. 4b, c). *OX11* gene expression is also induced by cold, by osmotic stress and by heat (data not shown). It is therefore possible that OX11 is involved in many more AOS-dependent processes and might act as a universal mediator of oxidative bursts and stresses in *Arabidopsis*. □

Methods

Plant growth conditions

Arabidopsis thaliana plants were grown on 1 × MS medium and 0.8% w/v agar or in peat plugs at 21 °C under 16-h light conditions at 60 μM m⁻² s⁻¹ light intensity. For infection with *P. parasitica*, plants on peat plugs were placed under a transparent dome at 16 °C under 8-h light conditions.

OX11::GUS reporter gene and OX11 complementation constructs

A 1,610-base-pair (bp) fragment containing the promoter and the first two nucleotides of the *OX11* coding sequence was amplified from genomic DNA by PCR with the primers 5'-GCGCGGATCCCGCTGGGATAATCTCAAAGG-3' and 5'-CGCGCTGCAGATAA TGTCGACGTTAGTAAAC-3', and cloned into the pDH51 plasmid²¹ containing the *GUS* reporter gene. The *OX11::GUS* fragment including a cauliflower mosaic virus terminator sequence was subsequently excised by restriction with *Bam*HI and *Kpn*I and ligated into the *Agrobacterium* binary vector pBIN19 (ref. 22). A 4,704-bp fragment containing the wild-type *OX11* gene as well as the 5' 2,148 bp and the 3' 1,173 bp was amplified from genomic DNA by PCR with the primers 5'-GCGCGAATTCTAAAGTGTAGGCGAATA GCTGGAGACT-3' and 5'-GCGCGGATCCGCTATCATTATTAGGAGAATGGGAG ATTG-3'. The fragment was cloned into the pFGC5941 binary plasmid by restriction with *Eco*RI and *Bam*HI and subsequent ligation. The resulting plasmids were transformed into *Agrobacterium tumefaciens*, strain C58C1 (ref. 23). Col-0, Ws-2 (wild types) and *ox11* mutant plants were transformed by the dipping method²⁴.

GUS staining

Seven-day-old seedlings were stained for GUS activity by submersion in staining solution (modified from ref. 25: 50 mM NaPO₄ pH 7.2, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 10 mM EDTA, 0.01% (v/v) Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid substrate in dimethylformamide (diluted from a 100 mM stock solution) and vacuum infiltration to 50 kPa for 3 min. Samples were incubated at 37 °C until the desired staining intensity was reached, and were destained in 80% ethanol. Images were taken with a Nikon Coolpix 990 digital camera mounted on a Leica DM R microscope.

Northern blot analysis

Northern analysis was performed as described⁸. Seven-day-old *Arabidopsis* seedlings were transferred into water and left to recover for 2.5 h. H₂O₂ or cellulase (Onozuka R-10; Yakult Ltd, Tokyo, Japan) was added to the indicated final concentrations. For wounding, about 25% of the cotyledon surface was damaged with tweezers. Control samples remained in water. Samples were frozen after 0.5, 1 or 3 h. *OX11* and β-TUBULIN (*TUB*)-specific probes were labelled with ³²P and hybridized to total RNA blotted onto nylon membrane.

Assessment of P. parasitica sporulation

Seven-day-old seedlings or 4-week-old plants on peat plugs were sprayed with a spore suspension of 5 × 10⁴ spores ml⁻¹. At 6 days after infection, 150–300 mg tissue (cotyledons and small leaves) from separate plugs was removed and weighed, and 200 μl H₂O was added. After vortex-mixing for 30 s, spores were counted on a haemocytometer. The mean of ten squares (each 0.1 mm³ suspension) was recorded per sample and samples were scored twice to ensure accuracy. The values were then converted to the number of spores per mg fresh weight²⁶.

Imaging and measurement of root hairs

Images of roots growing in air, away from the surface of solid growth medium (0.8% Phytigel; adapted from ref. 27) were captured with a Leica MZFLIII stereo binocular microscope, a SPOT RT colour digital camera, and SPOT Advanced software version 3.1 (Diagnostic Instruments). Root hairs that were in focus throughout their length were measured with Image J 1.29x (W. Rasband) and the results were analysed with Microsoft Excel 2000 and Minitab.

Immunocomplex kinase assays

Kinase activity assays were performed as described previously²⁸ on extracts from 3-week-old leaves. In brief, cleared cell extracts containing equal protein amounts were subjected to a 2 h preincubation in the presence of 20 μl mixed protein A–Sepharose and protein G–Sepharose beads (1:1). The supernatant was immunoprecipitated with 5 μg protein A-purified antibodies raised against synthetic peptides encoding the carboxy-terminal 7 or amino-terminal 15 amino acids of MPK6 and MPK3, respectively, and 20 μl of protein A–Sepharose beads. The antibody to OX11 kinase was raised against a synthetic peptide encoding the C-terminal 15 amino acids. The beads were washed three times with wash buffer and once with kinase buffer. Kinase reactions of the immunoprecipitated proteins were performed at 24 °C for 30 min in 15 μl kinase buffer containing 5 μg myelin basic protein, 0.1 mM ATP and 2 μCi [γ-³²P]ATP. The reactions were stopped by adding 4 × SDS loading buffer. Phosphorylation of myelin basic protein as substrate for MAPKs was analysed by autoradiography after SDS–polyacrylamide-gel electrophoresis.

Western blot analysis

Western blotting was performed with equal amounts (20 μg) of protein extracts separated by SDS–polyacrylamide-gel electrophoresis, immunoblotted to poly(vinylidene difluoride) membranes (Millipore) and probed with affinity-purified antibodies either in Tris-buffered saline pH 7.4 containing 0.05% Tween 20 for MAPKs or in phosphate-buffered saline pH 7.4 containing Tween 20 for OX11. Alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma) was used as secondary antibody and the reaction was revealed by fluorography (CDP-Star; Amersham Life Sciences).

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