

*B* expression: While in the eye-disc, the *B* and *dpp* express in spatially overlapping regions (see Figure 4 *b*), in the case of leg and antennal discs (Figure 4), there is only a limited overlap between the expression domains of these two genes. In spite of this limited overlap, the *Bar*-expressing annulus in *dpp* mutant discs was reduced and in the double mutants, it was completely disorganized. These suggest either a direct long-range field effect of Dpp on *B* expression or an indirect effect resulting from the altered expression of *wg*, *hedgehog* (*hh*) and other genes due to *dpp* loss-of-function mutation. This aspect is being examined further.

As has been reported earlier<sup>26</sup>, most of the *B*<sup>+</sup>; *dpp*<sup>d6</sup>/*dpp*<sup>d12</sup> males were completely devoid of external genitalia while a few had abnormal external genitalia. As in legs and antenna, the *Bar* mutation had an enhancing effect on male genitalia also since the external genitalia were absent in all *B*; *dpp*<sup>d6</sup>/*dpp*<sup>d12</sup> male flies (not shown). Interestingly, the external genitalia were not much affected in female flies of any of the genotypes. The enhancing effect of *B* mutation on male, but not female, external genitalia in *dpp* mutant background also warrants further study.

The classical view has been that the *Bar* locus has a function only in eye differentiation in view of its phenotypic effect being restricted to differentiation of ommatidia in eyes. Recently, this gene was shown to also function in the differentiation of the notal region of wing of *Drosophila*<sup>8</sup>. We have now shown that the *Bar* locus has roles in differentiation of legs and antennae (and possibly also the external male genitalia) as well. Thus it appears that this complex locus of homeo-box containing genes plays a much wider role in differentiation of different structures in *Drosophila*.

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## Isolation and characterization of PR1 homolog from the genomic DNA of sandalwood (*Santalum album* L.)

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**Genomic library was constructed using nuclear DNA prepared from tender leaves of sandalwood. Subsequently, screening with heterologous probes we could isolate the PR1 genomic homolog. Restriction mapping and hybridization experiments were carried out to obtain the coding region for PR1 gene. A 750 bp EcoRI fragment thus obtained was subcloned to yield pSaPR1, which was compared with the related sequences. Southern hybridization with genomic DNA digests was carried out to check its genomic organization. The induction of this gene was observed in the somatic embryos treated with salicylic acid, thereby implying its possible involvement during systemic acquired resistance.**

SELF defense in plants stems from the necessity of their survival against various pathogens. As observed, plants are being challenged constantly by various pathogens but disease is not always the inevitable outcome. Depending on the pathogen, plants exhibit different types of defense responses which can be classified into three classes according to their spatial and temporal occurrences<sup>1</sup>. The first class comprises immediate, early responses that involve changes in ion fluxes across the plasma membrane, synthesis of active oxygen species (oxidative burst)<sup>2-4</sup> and the hypersensitive reaction (HR). HR is characterized by a local necrotic lesion that effectively traps the pathogen to the site of infection and prevents its spread throughout the rest of the plant<sup>4</sup>. The second line of defense, thought to restrict the growth and development of pathogen, is activated at the site of infection. This response involves the *de novo* synthesis of several proteins including enzymes involved in phenylpropanoid metabolism, and the biosynthesis of phytoalexins and pathogenesis-related (PR) proteins. The third line of defense that can occur in many plant-pathogen interactions is triggered on in the non-infected parts of the plant, which is known as systemic acquired resistance (SAR). SAR is characterized by the protection of uninfected parts of a plant against a second infection by the same or even unrelated

pathogen<sup>5,6</sup>. SAR implies the existence of a signal molecule produced in the infected tissue, that moves throughout the plant to activate resistance<sup>7</sup>. Salicylic acid (SA) has been proposed to have a central role as a signaling molecule leading to SAR as its concentration rises dramatically after pathogen infection<sup>8-13</sup>. Furthermore, exogenously applied SA leads to typical SAR responses such as increased resistance to viral infection<sup>5,14,15</sup>. Recent evidence suggests that SA may not be the primary long-distance SAR inducing signal and that the production of this systemic signal is not dependent on SA accumulation. However, SA is required in uninfected tissues for transduction of the translocated signal into gene expression and resistance<sup>16</sup>.

SAR is associated with the systemic *de novo* synthesis of a large number of PR proteins. Their time of appearance and the known function of at least some of the PR proteins suggest their involvement in SAR. Some members of the PR family, chitinases and  $\beta$ -1,3-glucanases, inhibit fungal growth. Moreover,  $\beta$ -1,3-glucanases may release defense-activating elicitors. Direct evidence of the potential role of PR genes in plant defense has been obtained by the experiments which demonstrated that overexpressing PR genes can lead to enhanced resistance to certain pathogens<sup>17,18</sup>. Since PR genes are induced in parallel with the appearance of SAR, they are useful targets to develop protection strategies in plants. Moreover, in the systems where genetically defined resistance is not described or prior knowledge of pathogen avirulence gene is not available, development of disease resistance may be achieved by manipulating these sets of genes.

As part of a tree improvement programme we are trying to study the defense response in an economically important tropical timber tree sandalwood (*Santalum album* L.), which may be used later to develop disease-resistant plants. Towards this end, we have attempted to clone and characterize some of the PR genes from sandalwood. Here we report cloning and characterization of PR1 homolog from sandalwood genomic library. We could demonstrate the induction of this gene in the

somatic embryos when treated with salicylic acid, thereby implying its possible induction during SAR.

Somatic embryos used for this study were obtained by direct somatic embryogenesis (G. Lakshmi Sita *et al.* unpublished work) from internodal segments of young shoots. Briefly, explants were inoculated in MS medium<sup>19</sup> supplemented with thidiazuron (TDZ) and 6-benzyl-aminopurine (BAP) for direct somatic embryogenesis. Globular embryos thus obtained were transferred to MS medium supplemented with gibberellic acid (GA). Three- to 4-week-old somatic embryos were used for induction with SA and other downstream applications.

High molecular weight DNA was prepared from the nuclei isolated from sandalwood leaves. Briefly, sandalwood leaves were frozen and pulverized in liquid nitrogen which was then suspended in 5 volumes of cold nuclei isolation buffer (NIB) (15% sucrose, 50 mM Tris pH 8.0, 50 mM EDTA, 5 mM MgCl<sub>2</sub>, 5 mM mercaptoethanol, 150 mM NaCl). Once thoroughly mixed, the homogenate was allowed to pass through three layers of cheese cloth and then centrifuged at 1500 g for 10 min at 4°C. The precipitate thus obtained was resuspended in the same buffer containing 0.1% Triton X-100. After an incubation in ice for 10 min, a nuclear pellet was obtained by centrifugation at 100 g for 10 min at 4°C. This nuclear pellet was used for isolation of high molecular weight DNA by the standard procedure<sup>20</sup>.

DNA partially digested with *Sau3AI* was partially filled in and subsequently cloned in the λ GEM-11 half-filled arms as described in Promega Protocols. The genomic library containing 105 recombinants was screened with *Arabidopsis* PR1, PR2 and PR5 cDNA probes (kindly provided by Dr John Ryals, Navartis, USA). Out of 19 positive clones obtained after multiple rounds of screening, one that was positive for PR1 was taken up for further characterization. This genomic clone having the insert of size approximately 18 kb was subjected to restriction mapping followed by Southern hybridization to obtain the coding region for PR1. A 750 bp *EcoRI* fragment thus obtained was

subcloned in pBluescript to yield pSaPR1.

Sequencing was carried out using Sequenase Kit (ver 2.0) (USB Biochemicals, USA) following the manufacturer's instruction. The nucleotide sequence and the deduced amino acid sequence from the same is represented in Figure 1.

Genomic DNA was restricted with *HindIII*, *EcoRI*, *SacI* and *XhoI* which do not have an internal site within SaPR1 and fractionated in 0.8% agarose gels. DNA was transferred to nylon membrane (Hybond N<sup>+</sup>, Amersham Inc.) using TE-80 vacuum transfer system (Hoefer Scientific Instruments, USA). Blots were hybridized with SaPR1 probe. Probes were labelled with [ $\alpha$ -<sup>32</sup>P]dATP using Amersham's megaprime labelling kit. Hybridizations were carried out for 24 h at 42°C in 50% formamide, 5 × Denhardt's solutions, 6 × SSC, 1% SDS, 100 g ml denatured Salmon sperm DNA and blots

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gaattcatgccatgcacttccctttactccaattggctttctcaataggggaaaagccc
tccatttccgtcaaatgaccggcctcccctggctcttccaccgtccgggtctcttcc
tccctatgctatgctccccggcgaggcctaccacgcttgcgctgcggttttgcg
cagacggtctttccagtagtgccatcctccccgctgctgtatggcggttttccctt
M G G F S L
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A S A F L L S L W I T G T I V S S R A Q
aatagcgcaagattattcaacgggtcccaacgtgaggcagtgagggtgtagagataacc
N S A Q D Y S T V P N V R A V G V E I T
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P W D E Q R L A A S A R Q R A S D L K T
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R C R L V H S H S P Y G E N L A I T S G
cactttactaccttctcgccttccctcccctatggtggttgcgagaaagttaactacgcc
H F T T F L A F L P M W V V E K F N Y A
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A T L C V C H Q A A L R G C A R K S N G
ggaaccaagcaggccagcagcaatggaccgactggagtggttccgtactagcgcactcggc
G T K Q A S S N G P T G V V P Y *
gtcagcgcttctgcccactggagtgatccaataigtggttccgcacgttccaccagaattc

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**Figure 1.** Nucleotide and deduced amino acid sequences of the SaPR1. The genomic fragment contains 725 nucleotides. The stop codon is marked with an asterisk.

were washed finally at 0.1 × SSC, 0.1% SDS for 30 min at 65°C.

Total RNA was isolated from sandalwood somatic embryos (treated with SA or mock treated with water) by the GITC-acid phenol extraction method as described by Chomczynski and Sacchi<sup>21</sup>. 10 µg of total RNA was denatured in formamide,

separated by electrophoresis through formaldehyde agarose gels and blotted to Hybond N<sup>+</sup> filters<sup>22</sup>. Blots were hybridized with SaPR1 probe. Probes were labelled with [ $\alpha$ -<sup>32</sup>P]dATP using Amersham's megaprime labelling kit. Hybridizations were carried out in the same conditions as described earlier. Filters were washed finally at 0.5 × SSC, 0.1% SDS for 15 min at 50°C. All RNA gels were routinely visualized with ethidium bromide staining and equal loading was confirmed by reprobing the same blot with RNA gene probe.

The sequence of the predicted amino acids was aligned with related sequences obtained from SWISS-PROT data base. When compared with the known PR1 sequences it reveals 37–49% homology in the coding region (Table 1). Organization of SaPR1 sequences in the genome was made by genomic blotting experiments. Genomic DNA was cut with *EcoRI*, *HindIII*, *XhoI*, *SacI* restriction enzymes which do not cut within the 750 bp *EcoRI* fragment used as probe. As shown in Figure 2, the probe hybridizes predominantly to single fragments at high stringency in all four digests. In addition to this, other weak bands could be detected in some of the digests. To determine whether these fragments were due to partial digestion of DNA, the same digests of DNA were probed with other control DNA and the banding patterns indicated complete digestion (data not shown). Together, these results suggest that SaPR1 may react with other members of this gene family.

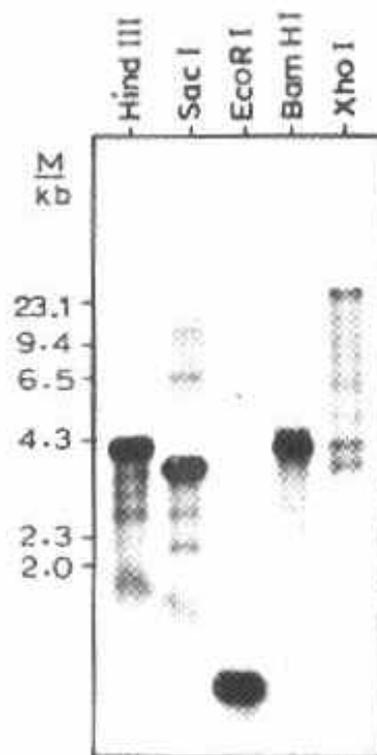
Expression of PR1 was checked in the somatic embryos mock treated with water and upon treatment with SA. Figure 3 shows that there is an induction of PR1, when the embryos were treated with SA. The probe hybridizes specifically to a single mRNA species of size 0.8 kb. Longer exposure of

the blot results in the appearance of faint signals in the uninduced lanes (data not shown). This corroborates well with the other reports of PR1 induction in various plants, thus suggesting the possible involvement of SaPR1 during SAR.

In summary, a genomic library was constructed from an economically important tropical timber tree, sandalwood and screened with *Arabidopsis* PR1, PR2 and PR5 cDNA probes. PR1 reading frame was identified from a genomic fragment, which codes for a putative protein of 143 amino acids and an estimated molecular mass of 15 kDa. The *SAR* gene shares 43–47% amino acid sequence identity with various *PR1* genes. Southern hybridization was carried out using SaPR1 probe to show its possible homology with other members of the gene family. Thus we have successfully demonstrated that this gene is induced by SA, and hence probably during SAR, by northern blot analysis.

**Table 1.** Percentage of identity of aligned amino acid sequences of PR1

	Tobacco PR1c	Tobacco PR1a	Tobacco PR1b	<i>Arabidopsis</i> PR1	Maize PR1	
<i>SaPR1</i>	47.9	47.1	46.3	45.4	36.7	Per cent identity amino acid overlap
	121	121	121	119	109	



**Figure 2.** Southern hybridization analysis. Genomic DNA (10 µg per lane) was digested with the indicated restriction enzymes. The migration of the molecular weight standards are indicated.

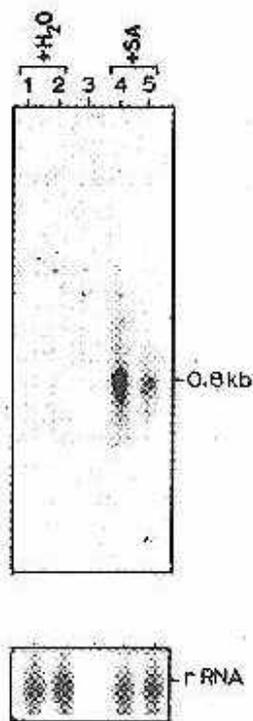


Figure 3. Expression of *SaPR1* transcript in SA-treated somatic embryos. 10 µg of total RNA on each lane from uninduced and induced embryos was loaded as indicated. Upper panel: shows the expression of *SaPR1* transcript in the induced embryos. The same blot was reprobed with rRNA gene as shown in the bottom panel. Lanes 1 and 2, RNA from water-treated embryos in different periods; Lane 3, RNA from control (no treatment) embryos; Lanes 4 and 5, RNA from SA-treated embryos in different periods.

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