show enrichment above the WHO guideline value in some places, mainly in the tube wells located near the ash ponds 4A, 4B and 1A (Table 7). Excess amount of arsenic and manganese affects the cardiovascular system, gastrointestinal tract, kidney, liver, skin and blood and prostrate. Excess amount of lead could lead to dysfunctioning of the kidney, gastrointestinal tract and respiratory systems due to inhalation of fine ash particles rich in lead.

The present study shows many potential environmental hazards related to coal combustion. Hence proper measures should be taken to check the release of toxins from the ash pond and subsequent mixing with the groundwater. A remedy is to have underground lining in the ash ponds to prevent direct contact of the ash pile with the top soil and the local drainage bodies. Use of bricks from fly ash meant for dwellings should be curtailed in the absence of detailed studies on the indoor radiation dose and its effect on the inhabitants for prolonged exposure. The radiation dose from thermal power plants can be reduced by reducing the ash content of coal and establishing thermal power plants away from populated area.

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Identification of elicitor-induced PR5 gene homologue in *Piper colubrinum* Link by suppression subtractive hybridization

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***Piper colubrinum* Link., the exotic wild *Piper* shows high degree of resistance to fungal pathogens and is a potential source of resistance genes. A PCR-based suppression subtractive hybridization (SSH) was used to identify *P. colubrinum* genes that are differentially expressed in response to the signalling molecule, salicylic acid (SA). A subtracted library of SA-induced genes was synthesized and one of the clones showed sequence homology to osmotin, a member of class-V group of pathogenesis-related (PR) gene family. The 315 bp gene fragment was used to probe total RNA prepared from untreated and SA-treated leaf tissues. Osmotin fragment cloned from the subtracted library was also used to probe RNA from ethylene-treated leaf tissues. Northern blot analysis revealed that osmotin is dominantly expressed in SA/ethylene-treated tissue. This indicates that SSH can be used to identify and clone PR genes in *P. colubrinum*.**

PIPER colubrinum Link. is an exotic wild species of *Piper*, which shows high degree of resistance to many devastating

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diseases of cultivated pepper, including foot rot and slow decline diseases caused by *Phytophthora capsici* and *Rhadopholus similis* respectively[1]. It has good potential as a donor plant in breeding programmes for the improvement of the cultivated species, *Piper nigrum*. Biotechnological approaches such as molecular resistance breeding, would provide immense potential for the improvement of cultivated black pepper. Salicylic acid (SA), the phenyl propanoid derivative plays a key role in disease resistance and has been implicated as a signal in systemic acquired resistance (SAR)[2]. The present study was aimed at identifying and characterizing SA induction-specific defense-related genes from *P. colubrinum* by suppression subtractive hybridization (SSH) technique. Only limited reports are available for *P. colubrinum* and we report here a molecular study for elucidation of the resistance mechanism operational in the plant.

A five-year-old plant of *P. colubrinum* maintained at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram served as the mother plant for the present study. Fresh cuttings made from the mother plant were planted in pots, and maintained under uniform conditions. Second leaves of fresh sprouts, which appeared after four weeks, were used for further experiments. For SA treatment, fresh leaves were washed well with Teepol solution followed by several rinses in sterile distilled water. After drying on crude filter paper, a constant weight of leaf tissue was cut into discs of approximately 1 cm^2 size and floated on 0.1 mM SA. The samples were incubated at room temperature for 24 h with constant shaking at 150 rpm in a shaker incubator. Ethylene in the form of ethephon was prepared in sterile water at a concentration of 1 mg/ml. Young leaves of potted plants of *P. colubrinum* were sprayed with the solution and covered with sterile plastic bag. Leaves were harvested after 12, 24 and 48 h of treatment. Equal quantity of fresh untreated leaf tissue served as control.

RNA isolation was performed using 800 mg each of control and SA-treated samples using Trizol (Invitrogen) reagent. Poly A mRNA was isolated from the samples using PolyATtract mRNA isolation kit (Promega) and finally dissolved in 4 µl RNase free water. mRNA isolation was proceeded by 1st and 2nd strand cDNA synthesis followed by subtraction procedure using PCR Select cDNA Subtraction kit (CLONTECH). A complete control subtraction was performed in parallel with the experimental subtraction using skeletal muscle cDNA provided as driver. The skeletal muscle tester cDNA was prepared by mixing control skeletal muscle cDNA with diluted ϕX 174/*Hae*III control DNA provided in the kit. For experimental subtraction, the double-stranded cDNAs synthesized from 2 µg mRNA from control and treated samples were digested with the restriction enzyme *Rsa*I. The control double strand cDNA was denoted as 'driver' and the SA-treated leaf tissue as the 'tester'. After *Rsa*I digestion to generate blunt-ended fragments, the tester double-stranded cDNA was divided into two portions and each was ligated with a different adaptor provided in the kit. The driver was not adaptor-ligated. This was followed by two hybridization reactions. The last step was a selective PCR amplification that could amplify only those fragments which had two different adaptors. After performing primary and secondary PCR steps for the preferential exponential amplification of differentially expressed sequences in the tester sample, the subtracted products were checked on a 1.2% agarose/EtBr gel. The bands were eluted using GFX Gel band purification kit (Amersham) and cloned into T/A cloning vector (pGEM-T Easy Vector System, Promega). The cloned products were transformed in *Escherichia coli* JM109 competent strain and positive transformants were selected by blue/white screening. Randomly selected positive clones were checked for the presence of insert fragments by performing PCR amplifications using subtraction-specific adaptor primers (provided in the kit). Selected positive clones were sequenced in an ABI 310 Prism automated sequencer. The sequencing primers used were SP6 5′-CATACGATTTAGGTGACACTATA-3′ and M13 Forward 5′-GCCAGGGTTTTCCCAGTCACGA-3′. For analysis of subtraction efficiency, the housekeeping gene, actin from *P. colubrinum* was amplified using 3′ and 5′ primers (Forward 5′-TCCATAATGAAGTGTGATGT-3′ and Reverse 5′-GGACCTGACTCGTCATACTC-3′). PCR was performed on subtracted and unsubtracted secondary PCR products with the actin primers. The samples were analysed after 18, 23, 28 and 33 cycles of PCR.

Sequence comparisons with the GenBank databases were performed using the BLAST programme of NCBI and sequence alignment was done using BioEdit software.

For Northern analysis, 20 µg total RNA from control and SA/ethylene-treated samples (2–48 h) was separated on 2% agarose–formaldehyde denaturing gel, run in 1X MOPS

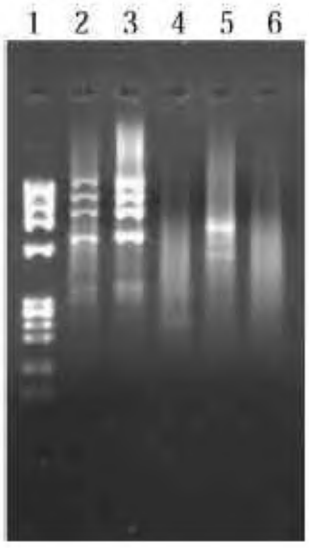

Figure 1. Secondary PCR products after subtraction. 1, Marker; 2, Control subtracted sample from kit; 3, Subtracted control skeletal muscle cDNA; 4, Unsubtracted control; 5, Subtracted sample; 6, Unsubtracted sample control.

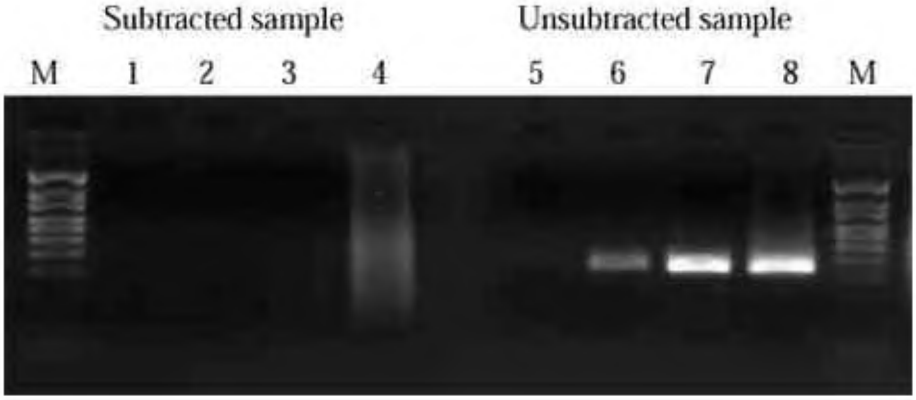

Figure 2. PCR analysis of subtraction efficiency using actin primer. M, Marker; lanes 1 and 5, 18 cycles; lanes 2 and 6, 23 cycles; lanes 3 and 7, 28 cycles; lanes 4 and 8, 33 cycles.

buffer and blotted onto a nylon membrane with 3 M NaCl/ 0.01 N NaOH. Hybridization was conducted in Church–Gilbert buffer (0.5 M sodium phosphate buffer, 7% SDS, 0.01% BSA and 1 mM EDTA at 65°C overnight). Membrane was washed with 1X SSC/0.1% SDS at room temperature and 65°C respectively, for 10 min followed by three washes with 0.5XSSC/0.1% SDS at 65°C. DNA probe consisted of a 315 bp long osmotin-like cDNA fragment obtained from the subtracted library and labelled by incorporating [α-^{32}P] dCTP in the PCR amplification step. The Northern experiments were repeated and gave consistent results.

The RNA appeared undegraded on 2% agarose gel. Typical A260/A280 absorbance ratios of the control and tester samples ranged from 1.8 to 2, with mean yield of 0.6 mg/g fresh weight. The A260/A280 ratios of purified poly A RNA of both samples were greater than 1.9. A clear smear greater than 0.5 kb was present on the 2% agarose gel, indicating that the quality of the obtained poly A RNA was sufficient for further use. The subtracted products on gel analysis appeared as a smear ranging in size from 200 bp to 2 kb, with 2 to 4 definite bands (Figure 1, lane 5), clearly distinguishing them from the unsubtracted sample control. The subtracted skeletal muscle sample (Figure 1, lane 3) showed DNA fragments corresponding to the ϕX174/*Hae*III digest (Figure 1, lane 1-Marker). The control-subtracted sample provided in the kit (Figure 1, lane 2) gave an identical pattern, which confirms the efficiency of the control subtraction experiment. Analysis of subtraction efficiency of experimental samples by PCR amplification of constitutively expressed actin gene primers revealed that it appeared after 23 cycles, when using the unsubtracted sample control as a template (Figure 2, lane 6), but did not appear until after 33 cycles when using the subtracted cDNA as a template (Figure 2, lane 4). This indicates that cDNA homologous to both tester and driver was eliminated by subtraction.

About 130 clones were obtained and cDNA inserts of randomly selected subtracted clones were PCR-amplified using the adaptor primer pair of the subtraction. Plasmid DNAs of 20 clones were purified and subjected to sequence determination. More than a quarter of the clones yielded sequence homology to known stress-related genes. Among them, a 315 bp fragment showing 67% homology to *Vitis vinifera* thaumatin-like protein and 61% homology to *Hordeum vulgare* thaumatin-like protein and *Oryza sativa* pathogenesis-related thaumatin-like protein mRNA was obtained. For Northern blots, 20 µg each of total RNA from control and 24 h SA-treated leaf tissue were hybridized with a probe of osmotin partial gene fragment (315 bp) obtained from the subtracted cDNA library. The probe hybridized to only the lane containing SA-treated sample RNA, while the untreated control did not show any signal (Figure 3). A time course experiment was also performed varying salicylic acid treatment time from 0 to 12 h (Figure 4). The expression profile of the osmotin homologue represented a wide range in transcript abundance between control (0 h) and 12 h SA-treated samples. The control sample did not show osmotin expression (Figure 4, lane 1), whereas 2 to 8 h SA-treatment showed a gradation in osmotin expression (Figure 4, lanes 2 and 3). The expression level peaked at 8 h and stabilized at 12 h (Figure 4, lane 4). Ethylene-treated samples also showed a progression in expression level of osmotin from control to 48 h treatment. While control untreated samples failed to exhibit detectable levels of osmotin, the levels of expression gradually peaked from 12 h, reaching a maximum at 48 h (Figure 5).

SSH technique combines a high subtraction efficiency with an equalized representation of differentially expressed sequences, which is achieved by suppression PCR[3]. Suppression PCR permits the exponential amplification of cDNA that differs in abundance, whereas amplification of sequences of identical abundance in the driver and tester populations is suppressed. This technique has been efficiently used for identification of many differentially expressed genes from plants[4,5]. We constructed a subtracted cDNA library with SA-treated leaf sample as tester and fresh untreated leaf sample as driver using SSH to identify genes specifically expressed or overexpressed by SA induction.

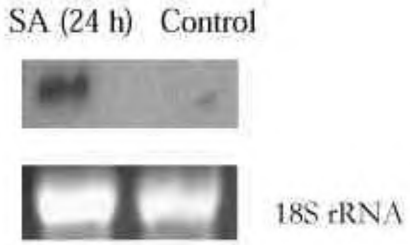

Figure 3. Northern blot probed with osmotin gene homologue.

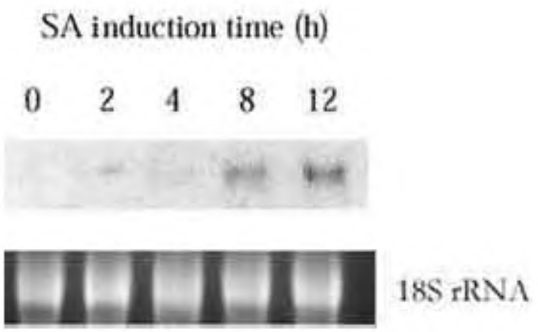

Figure 4. Northern blot – salicylic acid induction on timescale.

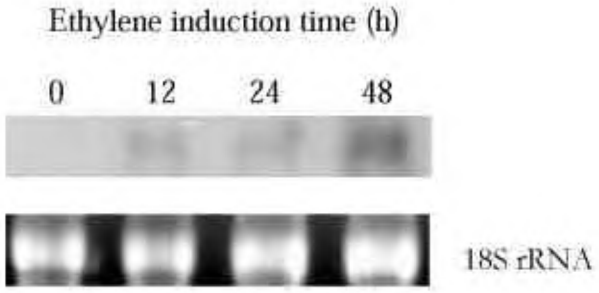

Figure 5. Northern blot – ethylene induction on timescale.

Our results indicate that in using SSH, differentially expressed genes of *P. colubrinum* resulting from SA treatment are enriched in the subtracted library. Northern blot analysis revealed that the subtraction was effective and confirmed the differential expression of a clone homologous to *Vitis* thaumatin-like protein (osmotin/PR 5). Based on expression, response and structure analysis, osmotin has been classified as a member of type-5 plant pathogenesis (PR)-related protein. This protein has a dual function: response to plant pathogenesis and to osmotic stress[6]. There is accumulating evidence that osmotin and osmotin-like proteins have *in vitro* anti-fungal activity, causing lysis of sporangia and growth inhibition of *Phytophthora infestans*[7]. Abiotic elicitors as well as microbial pathogens, including *P. capsici* were shown to strongly and preferentially induce osmotin (PR 5) transcripts in *Capsicum annuum*[8]. SA is one of the known inducers of the osmotin gene and is known to induce pathogen resistance in tobacco[9]. It was demonstrated to induce activation of osmotin promoters in potato[10]. Systemic acquired resistance has also been shown to be mediated by jasmonate or ethylene, which also reportedly act as wound signals in defensive response to insect attack[11]. Hence we sprayed intact leaves with ethephon, without causing physical injury. Osmotin probe derived from the subtracted library gave strong hybridization signals with total RNA prepared from ethylene treated leaves of *P. colubrinum*, which further substantiates the efficiency of subtraction. The results point to the suitability of SSH in identifying genes in the disease-resistance pathway. This is the first report of analysis of differentially expressed transcripts in *Piper* sp. by SSH technique. Clones from the enriched subtracted library will be particularly valuable because they are genes which play a role in stress and defence, and may be used in future as an important source of genes for improvement of cultivated black pepper. Our next step is to screen the entire subtracted library to identify more genes specifically induced by SA.

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