

エンドウ・PALキメラ遺伝子プロモータを導入したトランスジェニック・タバコにおける菌類感染および傷害に対する発現の解析

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著者名	Sriprasertsak,P. Salamah,A. 井村,善之 一瀬,勇規 白石,友紀 山田,哲治
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Expression of the Chimeric Pea *PSPAL2* Promoter in Transgenic Tobacco in Response to Fungal Ingress and Injury

Permpong SRIPRASERTSAK^{*,**}, Andi SALAMAH^{*}, Yoshiyuki IMURA^{*},
Yuki ICHINOSE^{*}, Tomonori SHIRAIISHI^{*} and Tetsuji YAMADA^{*}

Abstract

The 5'-upstream region of the pea phenylalanine ammonia-lyase gene2 (*PSPAL2-FL*, -2196 to +110) promoter was dissected by deleting in a series and fused to the coding region of the reporter gene encoding β -glucuronidase (GUS). The constructed chimeric promoters designated as *PSPAL2-FLd1* (-1486 to +110), *PSPAL2-FLd2* (-966 to +110), *PSPAL2-FLd3* (-594 to +110) and the full-length promoter *PSPAL2-FL* (-2196 to +110) were used to transform tobacco plants by the *Agrobacterium*-mediated leaf disk method. Histochemically, GUS expression of the *PSPAL2* promoter was examined in mature leaves of these transgenic tobacco leaves inoculated with pathogenic and non pathogenic fungi. GUS expression was induced by inoculation with a non pathogen, *Phytophthora capsici*, as detected by a very clear expression zone around the hypersensitive response (HR) area, especially in the transformants of *PSPAL2-FL* and *PSPAL2-FLd1*. However, on leaves inoculated with a pathogen, *P. nicotianae*, only weak and hazy colorations were detected around the inoculation site even in the transformant of *PSPAL2-FL*. Moreover, wounding mature leaves with a sterile razor blade also triggered remarkable expression around the wounded sites, especially in the transformant of *PSPAL2-FL*. We suggest that *PSPAL2* promoter expression in transgenic tobacco is induced not only by fungal ingress but also by wounding.

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Key words : *Pisum sativum*, phenylalanine ammonia-lyase gene2 (*PSPAL2*), promoter activity, *Phytophthora nicotianae*, *Agrobacterium*-mediated transformation, transgenic tobacco.

INTRODUCTION

Higher plants acquired defensive systems for survival under versatile environmental stresses. The phenylpropanoid biosynthetic pathway provides precursors for the elaboration of a variety of compounds with different functions. These include structural components of cell walls, such as lignins which impregnate xylem cell walls during differentiation, and suberin which is an integral constituent of the cell wall matrix in endodermal and phellogen tissues, and a wide variety of natural products such as anthocyanins, phytoalexins, absorbent of ultraviolet (UV), and cytokinin-like substances⁹⁾. A great number of genes encoding enzymes involved in the biosynthesis of phytoalexins, genes encoding other enzymes such as glucanase and chitinase that are antagonistic to pathogens, and genes associated with the deposition of structural polymers such as lignin and cell wall hydroxyproline-rich glycoproteins, have been investigated in relation to resistant responses induced by

biotic or abiotic elicitors^{1,4,6,7,25)}. The activation of these defense-related genes after inoculation with fungal or bacterial pathogens has also been shown in a number of plant-pathogen interactions^{12,21,26)}.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is one of the key enzymes that are essential for defense reactions in plants. PAL catalyzes the first step of the general phenylpropanoid metabolism, the deamination of L-phenylalanine to produce cinnamic acid, and regulates subsequent reactions leading to the production of a wide variety of natural products. These essential roles of isoflavonoids in plant development and defense responses against various environmental stresses, including pathogen ingress, and the structural and regulatory function of phenylalanine ammonia-lyase (*PAL*) genes, have been extensively studied in various higher plants such as alfalfa¹³⁾, *Arabidopsis*^{30,36,46)}, carrot⁴³⁾, French bean^{37,39)}, *Lithospermum erythrorhizon*⁵⁰⁾, parsley²⁹⁾, pea⁴⁹⁾, pine⁴⁷⁾, poplar⁴²⁾, rice^{34,51)}, sweet potato⁴⁴⁾, tobacco¹⁰⁾ and tomato²⁷⁾.

In pea, genes encoding phenylalanine ammonia-lyase

* Laboratory of Plant Pathology and Genetic Engineering, College of Agriculture, Okayama University, Tsushima Okayama 700-8530, Japan 岡山大学農学部

** Present address : Central Laboratory and Greenhouse Complex, Kasetsart University Research and Development Institute, Kasetsart University, Kamphaengsaen, Nakhon Pathom 73140, Thailand 現在 : タイカセサート大学

(*PSPAL*) form a small multigene family comprised of at least three members²³. Two members of a *PSPAL* family, designated as *PSPAL1* and *PSPAL2*, were cloned and characterized⁴⁸. Both *PSPAL1* and *PSPAL2* were induced by fungal elicitors but suppressed by fungal suppressors produced by a pea pathogen, *Mycosphaerella pinodes* (Berk. et Blox.) Stone, a fungus pathogenic on pea⁴⁹. The structures of two suppressors from *M. pinodes* were determined and named as Suppressin A and Suppressin B⁴⁰. Moreover, it was shown that the putative receptor for these suppressors might be a protein that tightly binds to cell wall-bound ATPase or the ATPase itself²⁴.

Current molecular study of plant defense genes has been focused on the expression and regulation of promoters that are associated with the specific induction of certain enzymes and proteins. In most cases, the mechanisms have been analyzed by monitoring transient expression of a reporter gene of chloramphenicol acetyltransferase (CAT) in electroporated protoplasts and/or the expression of β -glucuronidase (GUS) in tissues of transgenic plants^{2,5,6,8,17,28,32,41}. Recently, the temporal and spatial expression pattern of the *PSPAL1* promoter was characterized in response to pathogen ingress and wounding in transgenic tobacco plants²³. The promoter dissection of *PSPAL2* has been performed in a transient transforming assay by electroporation of pea protoplasts, then determining their responses to environmental stimuli such as fungal elicitors, Suppressin or UV light irradiation⁴⁹. Functional analysis of 5'-nested deletions showed that an enhancer-like element is located in the TATA-distal region from -2196 to -406, in which the consensus sequence motifs known as box I, box II and box IV^{29,48} were present in close proximity.

The objective of the present study is to elucidate the role of the 5'-upstream region of pea *PSPAL2* promoter in responses of transgenic tobacco plant tissues to microbial invasion.

MATERIALS AND METHODS

Plant material and transformation *Nicotiana tabacum* cv. Xanthi NC (kindly provided by Japan Tobacco, Inc.) was used in the transformation experiments. Seeds were surface-sterilized in 5% hypochlorite for 10 min, followed by soaking in 70% ethanol for 10 min, and rinsing in sterilized water. Sterilized seeds were germinated in a sterile petri dish containing MS medium as described by Murashige and Skoog³⁵. A leaf disk of the sterilized tobacco was co-cultured in MS medium for 15–30 min with $1\text{--}5 \times 10^8$ cell/ml of *Agrobacterium tumefaciens* LBA4404³¹ carrying the specified chimeric genes as will be described. After drying on a sterilized Whatman 3MM filter paper to remove excess bacteria, inoculated leaf disks were placed abaxial surface down on MS medium containing NAA 0.1 mg/l, BA 1.0 mg/l. The inoculated leaves were incubated at 22–24°C for 2 days, then transferred onto MS-selective

medium containing the same concentration of NAA, BA and supplemented with kanamycin (100 mg/l) and crafuran (500 mg/l) until shoots formed. After 2–3 weeks of incubation, adventitious shoots were transferred to new MS medium and incubated until roots formed¹¹. All plant materials were incubated at 22–24°C under 16-hr light (150 $\mu\text{E}/\text{m}^2/\text{s}$), 8-hr dark conditions. Young seedlings were transplanted into soil and incubated in a greenhouse.

PAL-GUS chimeric gene fusions The pea *PSPAL2* full-length promoter (*PSPAL2-FL*) and the deleted chimeric promoters had been constructed into CAT reporter gene for analyzing the transient expression in electroporated protoplasts⁴⁸. To investigate the expression of *PSPAL2* promoter in transgenic tobacco plants, the *PSPAL2-FL* promoter and the three selected deletion construct promoters in the CAT reporter gene were amplified by PCR using specific primers and subcloned into pBluescriptII KS(+) at *Hind*III and *Bam*HI sites, then ligated to pBI101.2 in frame of GUS reporter gene. The *PSPAL2-GUS* chimeric promoter constructs were purified and transformed into *A. tumefaciens* LBA4404 by the freeze-thaw method¹⁸.

GUS-PCR and DNA manipulation Genomic DNA extraction for GUS-PCR was performed as described by Hosaka¹⁹. GUS-PCR was amplified using *GUS*-specific primers. DNA manipulation was performed according to the standard methods described by Sambrook *et al.*³⁸ or as specified by the manufacturer's protocols.

Histochemical GUS Assay Mature leaves were fixed by soaking in 1% formaldehyde in 50 mM sodium phosphate buffer (pH 7.0) for 10 min and rinsed three times with 50 mM sodium phosphate buffer (pH 7.0). Then, they were incubated in X-Gluc solution (1.0 mM 5-bromo-4-chloro-3-indolyl β -D-glucuronide) in 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA at 37°C overnight as described by Jefferson *et al.*²². Penetration and fixation of X-Gluc solution into tissues was facilitated by vacuum infiltration. Staining reactions were stopped by transferring the tissues into 70% ethanol.

Wounding and Inoculation Fungi used in this study were *Phytophthora nicotianae* van Breda de Haan var *nicotianae* Waterhouse, Po5 (*P. nicotianae*), a tobacco pathogen, and *P. capsici* Leonian IKEDA-1, a nonpathogen of tobacco. Fungal mycelia were scraped off PDA media and used to inoculate tobacco leaves which had been sterilized with 70% ethanol, at the site where a cross-cut was made with a sterile razor blade for aiding fungal invasion. The GUS expression of the transformants was histochemically detected as previously described.

RESULTS

Histochemical GUS expression

The translational *PSPAL2*-GUS fusion, *PSPAL2-FL* (−2196 to +110) and three deleted chimeric promoters designated as *PSPAL2-FLd1* (−1486 to +110), *PSPAL2-FLd2* (−966 to +110) and *PSPAL2-FLd3* (−594 to +110) were used to transform tobacco plants (Fig. 1). The integration of *PSPAL2*-GUS fusions in the genome of transgenic tobacco plants was confirmed by PCR

analysis using *GUS*-specific primers. Representative observations with at least three independent tobacco-transformant lines are shown in Plate I. The leaves of transgenic tobacco plants carrying *PSPAL2-FL* or chimeric promoter constructs were inoculated with *P. nicotianae*, a tobacco pathogenic fungi (P) or with *P. capsici*, a nonpathogen (NP). The results showed that histochemically detected GUS expression in transgenic tobacco plants was highest at 48 hr when incubated at 22–24°C and gradually faded at the hypersensitive response (HR) area around the inoculation site after 72

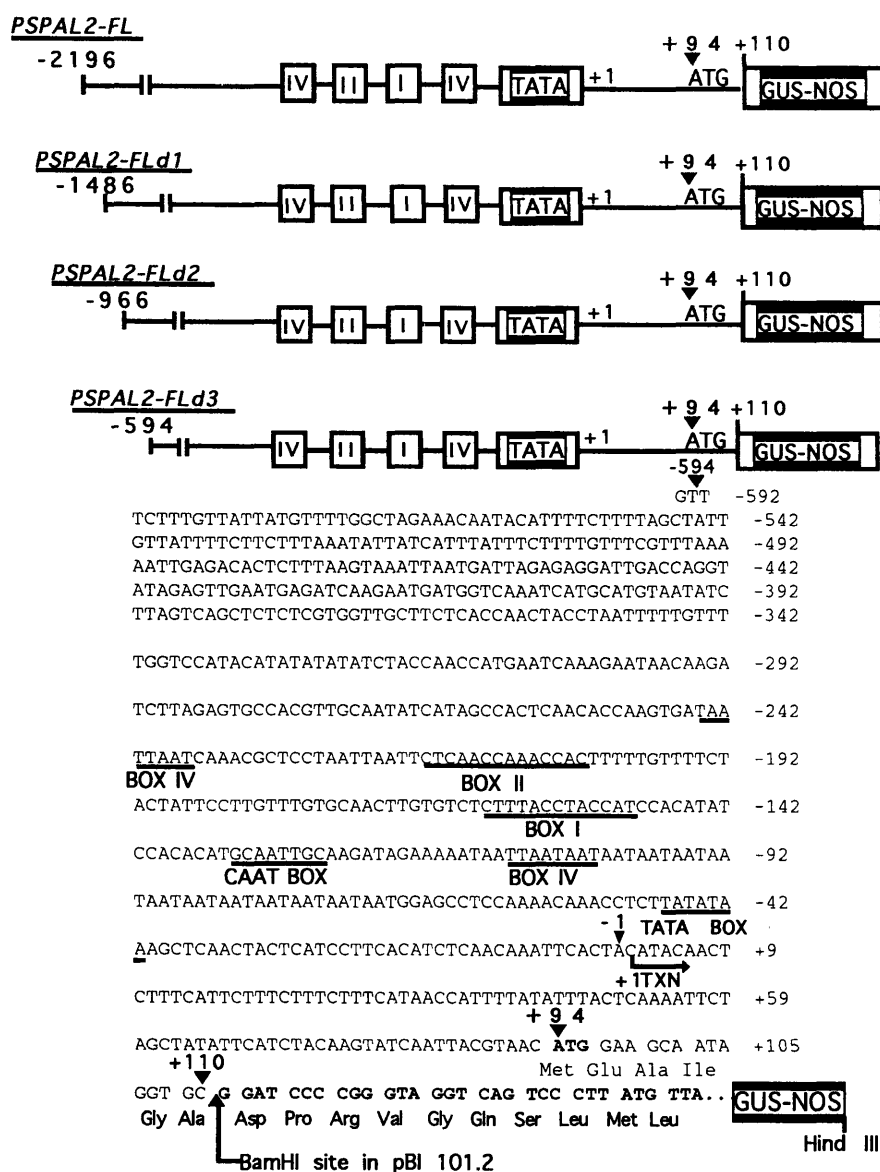


Fig. 1. Schematic representation of *PSPAL2* promoter-GUS-NOS fusions. Full-length (*PSPAL2-FL*) promoter-sequence and the three deleted chimeric promoter constructs (*PSPAL2-FLd1*, *PSPAL2-FLd2*, and *PSPAL2-FLd3*) were fused to GUS in pBI101.2 (Toyobo Inc., Kyoto, Japan) at *Hind*III and *Bam*HI sites. The type of fusions and the positions where GUS-NOS cassette was connected are schematically represented relative to a putative translation initiation codon (ATG) and transcriptional start site (TXN). The nucleotide sequences of all chimeric promoter constructs and the positions where GUS-NOS cassette were connected are shown and indicated with closed triangles. The numbers on top denote the nucleotide position from the transcriptional start point⁴⁹. Abbreviations: TXN and ATG, putative *PSPAL2* transcriptional and translational initiation sites, respectively; GUS and NOS, β -glucuronidase gene in pBI 101.2 and *A. tumefaciens* nopaline synthase gene terminator sequence, respectively.

hr of incubation (Plate I), where a plant defense system had presumably been established for blocking fungal invasion, in a manner similar to the expression of *PSPAL1* promoter²³⁾. The pattern of GUS expression after inoculation with a pathogen was not as clear as the one observed in the necrotic area after inoculation with a nonpathogen and never faded until the whole leaf was colonized.

Expression of deleted *PSPAL2* promoter after fungal infection

To discriminate the expression of the deleted *PSPAL2* promoter upon fungal infection, mature leaves of transgenic tobacco plants carrying the *PSPAL2* chimeric promoter constructs were inoculated with *P. capsici*. This nonpathogenic fungus induced a very large, clear GUS expression zone around the site of the hypersensitive response (HR), especially in the transformants of *PSPAL2-FL* (Plate II: A) and *PSPAL2-FLd1* (Plate II: B) at 48 hr after the inoculation. However, the responses to fungal infection were not as high in the transformants of *PSPAL2-FLd2* (Plate II: C); the expression zone was restricted to the area around the inoculation site. GUS expression in the transformant of *PSPAL2-FLd3* was almost negligible (Plate II: D). The results showed that the pea *PSPAL2* promoter expression in transgenic tobacco is strongly affected by the sequences in the 5'-upstream region as previously shown with transient CAT expression in electroporated pea protoplasts⁴⁸⁾. The functional analysis of 5' nested deletions of *PSPAL2* promoter in electroporated pea protoplasts showed that an enhancer-like element is located at the TATA-distal region from -2196 to -406, and this promoter was activated by the fungal elicitor from *M. pinodes* and partially suppressed by the suppressor from the same fungus⁴⁸⁾.

Interestingly, the GUS expression of our constructs upon fungal ingress in transgenic tobacco leaves demonstrated the induction of positive defense responses at the sites of infection at different levels depending on the additional sequences at the 5'-upstream region. Because we have observed a very low level of GUS expression in all the transformants of *PSPAL2-FLd3* compared to *PSPAL2-FL*, the lower level of GUS expression is not likely due to a position effect of the integration of the chimeric gene. This phenomenon may be explained by the lack of some active elements that are needed for the regulation of the *PSPAL2* promoter. These elements are likely to span from -966 to -2196. Moreover, we have observed GUS expression in mature transgenic tobacco leaves injured with sterile razor blade histochemically. Intense blue colorations were observed at wounded sites (W) and restricted adjacent areas. The GUS expression of the deleted *PSPAL2* promoter after wounding also declined from a high level to an extremely low one as the deletion was extended, in a manner similar to the expression after fungal ingress (Plate II: E-H).

DISCUSSION

In a previous study, we found that the pea phenylalanine ammonia-lyase gene2 (*PSPAL2*) promoter was induced by a fungal elicitor and suppressed by suppressors isolated from a pea pathogen, *M. pinodes*⁴⁹⁾. The expression of plant genes can be induced by environmental stresses such as wounding, UV irradiation, abiotic and biotic elicitors. For example, regulation of promoters of the *Arabidopsis STP4* gene (for sugar transport protein⁴⁾ under the stresses of wounding, elicitors, and pathogen challenge⁴⁵⁾ and of the melon ACC (1-aminocyclopropane-1-carboxylate) oxidase gene in transgenic tobacco plants under the stresses of wounding, pathogen attack and treatment with ethylene or copper sulfate³⁾ were also reported. Recently, the pea phenylalanine ammonia-lyase gene1 (*PSPAL1*) promoter was found to exhibit a temporal and spatial pattern of GUS expression in response to pathogen ingress and wounding in transgenic tobacco²³⁾. In this paper, we have for the first time demonstrated the expression of pea *PSPAL2* full-length promoter (*PSPAL2-FL*) and the three types of sequentially deleted chimeric promoter constructs designated as *PSPAL2-FLd1*, *PSPAL2-FLd2* and *PSPAL2-FLd3* in transgenic tobacco upon fungal ingress. We observed basal GUS expression in tissues of roots, stems and leaves during developmental *in vitro* growth of young seedlings before transplanting to soil. The levels of GUS activity in tissues of transgenic plants which had been transformed with an additional sequence at the 5'-upstream *PSPAL2* promoter were also determined. Extremely low GUS expression was observed in healthy or undisturbed mature leaves (data not shown). However, the *PSPAL2* promoter in leaves of transgenic tobacco plants after transfer to the greenhouse was induced upon fungal ingress, especially when leaves inoculated with *P. capsici* were incubated at 22-24°C for 48 hr. Marked expression was detected at the HR area surrounding the inoculation site of the transformant of *PSPAL2-FL* (Plate II: A). Extremely low GUS expression was observed in the transformant of *PSPAL2-FLd3* (Plate II: D) although brown necrotic regions developed at the cross cut (Plate II: D). The results demonstrated that the region from -966 to -2196 of *PSPAL2* promoter play a crucial role in the regulation of induction of GUS activities in the mature leaves of transgenic tobacco plants. Moreover, we have observed the induction of GUS expression in wounded mature leaves. The GUS expression in response to cut injury also declined in leaves transformed with the deleted promoter constructs in a manner similar to the expression after fungal ingress (Plate II: E-H). It is thus clear that the 5'-upstream region between +110 to -594, which contains important box sequences such as box I, box II and box IV as shown in Fig. 1⁴⁸⁾, was insufficient to establish the full capacity of defense gene response under stresses. The additional sequences from -594 to -2196 are

obviously necessary for the functional expression of the gene. The role of the 5'-upstream region in plant defense responses has been widely studied in terms of regulation in transgenic tobacco. The study of the significance of the 5'-upstream region of the pathogenesis-related protein (PR) 1a promoter which contains the flanking regions up to -906 and -1533 revealed its regulatory function in expression in transgenic tobacco under stresses of pathogen attack and abiotic elicitor. All tobacco transformants exhibited induced GUS expression after infection with tobacco mosaic virus or treatment with acetylsalicylic acid¹⁶). Recently, the link between the activation of retrotransposons and plant defense responses has been suggested in relation to possible genome restructuring that is needed in response to environmental fluctuations such as pathogen attacks and wounding^{14,15,33}). In our study, we have found retrotransposon-like sequences in the 5'-upstream regions of *PSPAL2* and of another elicitor-inducible defense gene, *PSCHS1* (chalcone synthase gene) in *Pisum sativum* : -2772 to -530²⁰). Interestingly, these putative sequence motifs are located in the region between -966 and -2196 in which we found higher GUS expression as a defense response to fungal ingress in transgenic tobacco plants. The role of active elements or other related factors that are involved in plant defense-related *PSPAL2* promoter expression under environmental stresses, especially the functional relationship to retrotransposon-like sequence, will be reported elsewhere.

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和 文 摘 要

Permpong SRIPRASERTSAK・Andi SALAMAH・井村善之・一瀬勇規・白石友紀・山田哲治: エンドウ・PAL キメラ遺伝子プロモータを導入したトランスジェニック・タバコにおける菌類感染および傷害に対する発現の解析

5'-PAL 遺伝子プロモータ上流領域 (-2196~+110) を順次削り取り, GUS レポーター遺伝子に連結したキメラ遺伝子をタバコに導入後, 4 種 *PSPAL2-FL* (-2196~+110), *PSPAL2-FLd1* (-1486~+110), *PSPAL2-FLd2* (-966~+110), *PSPAL2-FLd3* (-594~+110) のトランスジェニック植物体を作成した。トランスジェニックタバコ葉における GUS 発現様式を組織学的に観察したところ, タバコの非病原菌 (*Phytophthora capsici*) 接種において, *PSPAL2-FL* および *PSPAL2-FLd1* 導入体では, 接種 2 日目で過敏感反応を起こした組織の周辺細胞できわめて顕著な GUS 発現が検出されたのに対し, 病原菌 (*Phytophthora nicotiana*) 接種葉では, 微弱な GUS 発現が接種部位周辺に拡がって検出された。また, 剃刀でタバコ葉に付傷した場合にも顕著な GUS 発現がみられた。これらの結果は, *PSPAL2* の遺伝子発現は病原菌接種に限らず, 傷によっても誘導されること, 本発現にはプロモータ上流域 (-2196~-966) が必要であることを示している。

Explanation of plates

Plate I Histochemical GUS expression of *PSPAL2-FL* promoter upon wounding (W) and fungal ingress with a pathogen (P, *P. nicotianae*) and nonpathogen (NP, *P. capsici*) in transgenic tobacco leaves. Control treatment is shown as C.

A: 24 hr after inoculation or wounding

B: 48 hr after inoculation or wounding

C: 72 hr after inoculation or wounding

Plate II Expression of full-length (*PSPAL2-FL*: A, E) and deleted *PSPAL2* promoters (*PSPAL2-FLd1*: B, F; *PSPAL2-FLd2*: C, G; *PSPAL2-FLd3*: D, H) at 48 hr after wounding (W) and inoculation with a nonpathogen (NP, *P. capsici*) in transgenic tobacco leaves. Bars equal 1 mm.

Plate I

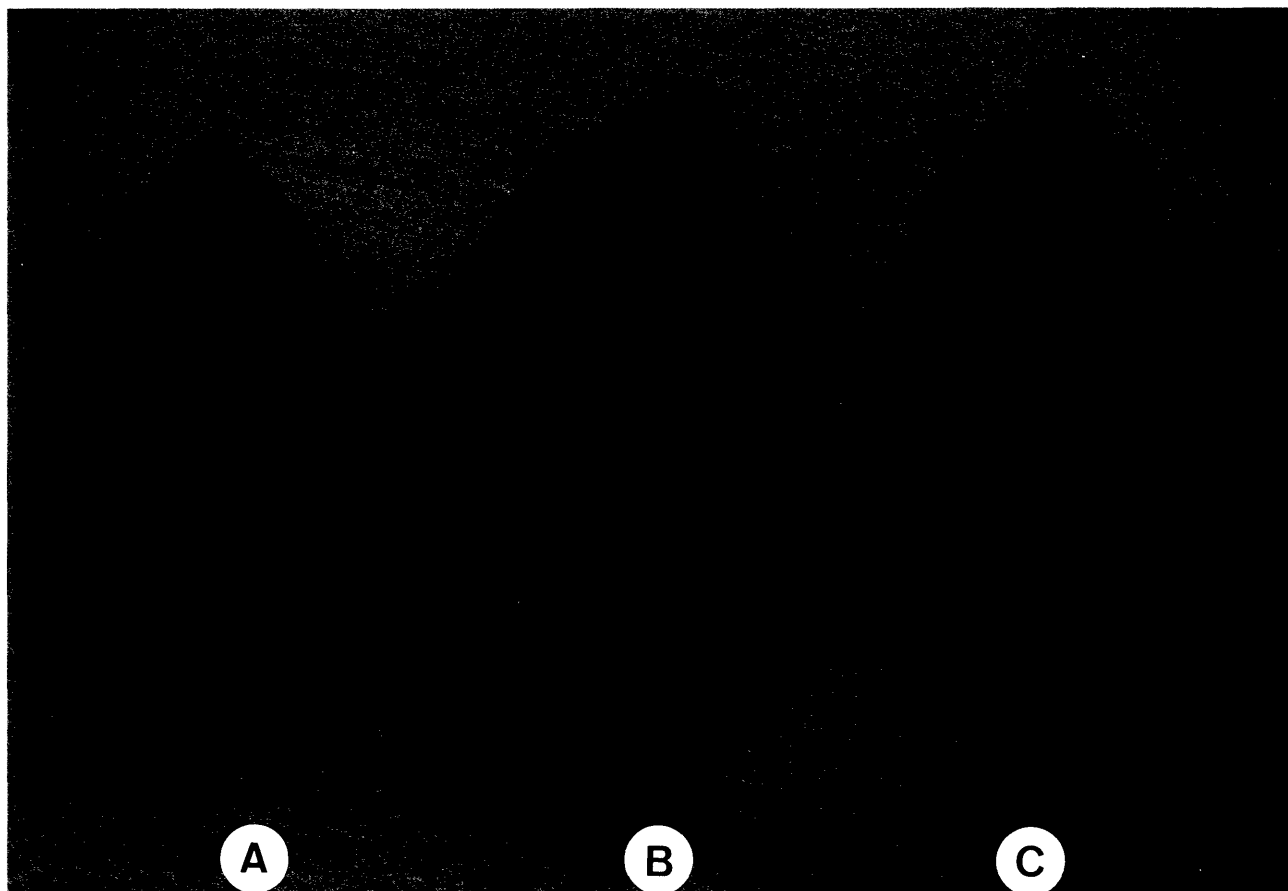


Plate II

