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Transgene Expression and Agronomic Improvement of Rice

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Transgene expression and agronomic improvement of rice

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[Plate 1]

SUMMARY

A reliable system for transformation and regeneration of rice protoplasts yielding fertile transgenic plants has been established. After co-electroporation of DNAs encoding a selectable marker and the gene of interest, protoplasts are regenerated to yield fertile plants. To date more than 70 different genes of interest have been successfully introduced and their patterns of expression are being studied. As in the case of dicot plants transformed by the Ti-plasmid vector approach, integration and expression appear to be stable in the transgenic monocots over several generations. Detailed comparative studies on gene expression in rice are underway using promoters for triosephosphate isomerase, a ubiquitously expressed gene encoding a cytosolic enzyme vital in the glycolytic cycle, two genes encoding members of the cyclophilin family, peptidyl-prolyl *cis-trans*-isomerases that are abundant in meristematic regions and are thought to participate in the correct folding of nascent proteins, and a gene encoding a tissue (root)-specific protein. Initial analyses suggest that the spatial expression of these genes in transgenic plants, using GUS reporter constructs, appears to be very sensitive to the nature of the 3′ flanking region present in the gene construct. Constructs containing a coding region for arcelin, a bean seed protein with putative anti-insecticidal properties, and others containing viral sequences that may provide novel approaches for protection against tungro and other viral infections have been introduced into rice plants.

1. INTRODUCTION

Alchemy and biotechnology evoke similar emotions in many people. How desirable to turn base metal into gold! How wonderful to adorn the world with beautiful things! How exciting it will be to use genes from vile bacteria and fungi to protect valuable crops! How wonderful to reduce the use of insecticides, to increase seed quality and create new ornamentals!

As rice is a major food source for millions of people in both developed and developing nations around the world, it is hardly surprising that great interest exists both in industrial and philanthropic organizations to develop biotechnological approaches for improvement of this crop. However, while *Agrobacterium tumefaciens*-mediated gene transfer has been well established over the past decade for dicot plants, economically important monocots such as maize, wheat and rice are not amenable to this approach. As an alternative, methods for direct introduction of DNA sequences into the genomes of these crops are being developed. In this article, we present results from our system for transformation and regeneration of fertile rice plants that is based upon electroporation of protoplasts from embryogenic suspension cultures.

2. THE REGENERATION SYSTEM

An overview of the regeneration system used is provided in figure 1. The first phase is the establishment of an embryogenic cell suspension; such cultures have been derived from calli initiated using either immature embryos (Battraw & Hall 1992) or immature panicles (modified from Li *et al.* 1992). Once established, the regenerability of a given suspension culture is checked, and the culture typically yields protoplasts suitable for transformation for about three months. After this period, regenerability of the protoplasts derived by enzymic digestion of the cell wall (Battraw & Hall 1991) decreases. However, older cultures are often used for nurse cells to assist in the recovery and regeneration of protoplasts after electroporation.

3. TRANSFORMATION PROCEDURES

Although we have had success with both electroporation and polyethylene glycol (PEG)-mediated transformation (Battraw & Hall 1991) of protoplasts, we have chosen electroporation as the standard approach. This is because electroporation conditions

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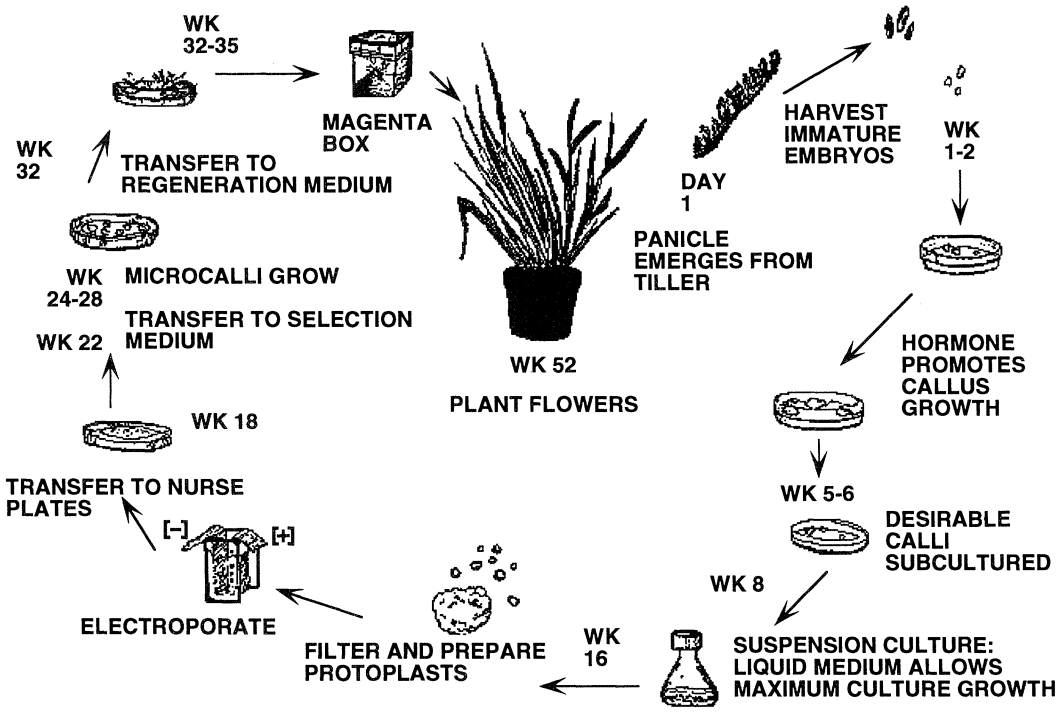

Figure 1. Cycle for regeneration and transformation of rice. Immature embryos are dissected as the panicle emerges from the tiller and used as the starting material for callus growth (day 1). After several subcultures, an embryogenic suspension is established from selected calli; some 8 weeks later (by week 16) the suspension cells have a doubling time of 2–3 days and are suitable for preparation of protoplasts for electroporation. As shown, it takes about 8 months from the date of electroporation to obtain the first flowering tiller.

can be closely standardized whereas different batches of PEG yield varying results and because successful transformation with PEG often results in a very high number of copies of the DNA sequences integrated.

The electroporation solution contains separate plasmids bearing the gene of interest (GOI) and the gene encoding the selectable marker. As indicated in figure 1, after electroporation, the protoplasts are plated on a filter disc over a nurse layer and cultured for about two weeks to give microcalli of 50–100 cells, prior to selection for transformants. We have found that the *bar* gene that encodes phosphinothricin acetyl transferase fused to a maize ubiquitin promoter and a *nos* 3′ transcriptional terminator (Toki *et al.* 1992) provides an excellent selectable marker. Using bialaphos ($1\ mg\ l^{-1}$) as the antagonist, no untransformed calli have escaped our selection procedure. After 4–6 weeks on selection, viable microcalli are transferred onto regeneration medium and subsequently placed in plastic regeneration boxes in the light (figure 1). Leaves from plants that are 3–5 cm tall are used for polymerase chain reaction (PCR) (Edwards, Johnstone & Thompson 1991) analyses to detect the GOI. GOI-positive plants (typically, some 20% of the bialaphos-resistant regenerants) are transferred to soil and, after an adaptation period of 4–6 weeks in an environmental chamber, adequate tissue is available to permit determination of the genomic arrangement of the GOI by Southern (1975) analysis. The plants are subsequently grown to maturity in a greenhouse adapted for culture of genetically engineered plants. Five generations of plants containing a neomycin phosphotransferase gene have shown normal Mendelian segregation, no rearrangements of the sequence following introduction, and levels of expression seen in first and second generation progeny (Battraw & Hall 1992) have been maintained. A summary of transformation experiments conducted in 1992 is presented as table 1.

4. GENE EXPRESSION STUDIES

Regulation of gene expression in transgenic dicots has been intensively studied during the past decade. Such research has led to the identification of domains responsible for spatial and temporal regulation of seed protein gene expression (Bustos *et al.* 1991) and many other features of gene function (Kuhlemeier 1992). Although relatively few examples of regulated gene expression are available for transgenic monocots, there are indications that substantial differences may exist from the generally accepted canons for dicots. For example, the presence or absence of introns in alcohol dehydrogenase and actin gene constructs has been shown to have a dramatic effect on expression levels (Callis *et al.* 1987; McElroy *et al.* 1990). Whereas anaerobic stimulation of ADH expression is typically thought to be confined to root tissues, expression and stimulation in transgenic rice leaves has been detected (Kyozuka *et al.* 1991; our own unpublished observations).

Studies on the temporal and spatial regulation of genes encoding rice seed proteins have been hampered by the infrequency with which progeny bearing mature seeds are obtained. However, initial reports indicate that the promoter for phaseolin, the major

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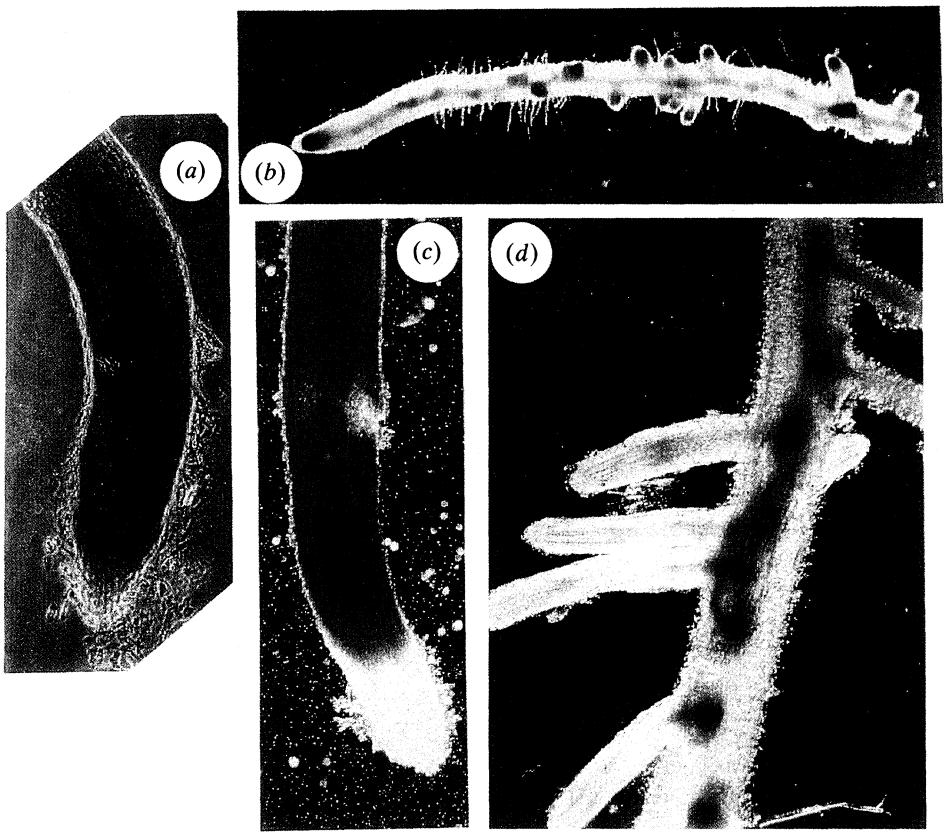

Figure 2. Comparison of root activity of *Cyp1* and *RCg2* genes in transgenic rice. (*a*) GUS expression (blue colour) of *Cyp1* in the root apex is especially strong in the meristematic and early differentiating regions. (*b*) This is again seen during the development of lateral roots where these areas express GUS, but little or no expression is seen in the differentiated tissues of the cortex. (*c*) Expression from the *RCg2* promoter can be seen in cells of the root cap and differentiated areas, but is essentially absent from the meristem and regions of early differentiation. (*d*) As lateral roots develop, activity is seen in the vascular areas of differentiated tissue. This direct comparison is useful in showing that the spatially distinct activities apparent for these promoters do not derive from penetration differences for the X–gluc substrate, or that they simply reflect cell size and cytoplasmic content.

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Table 1. *Summary of annual transformation experiments*

(The numbers shown indicate the scale of transformation and regeneration possible with a team containing two tissue culture scientists, a molecular biologist for preparation of DNA for electroporation and plant analysis by PCR and Southern techniques and a plant care (environmental chamber and greenhouse) technician. Not all of the plants indicated as containing the GOI will be capable of expression since deletions or rearrangements of flanking sequences may have occurred.)

electroporation experiments	21
different types of constructs electroporated	71
bialaphos-resistant microcalli produced	9601
plants regenerated	835
plants regenerated containing the gene of interest (GOI)	245

bean seed protein, does not function in rice although phaseolin protein can be expressed in transgenic rice from a wheat glutelin (Gt-1) promoter (Zheng *et al.* 1991). Given the difficulty in obtaining multiple transformants expressing seed-specific genes, we have initiated studies on a gene (RCg2) that encodes a root-specific protein. Surprisingly, although RNA blot analysis revealed strict spatial confinement of expression to rice roots, chimeric constructs containing the rice promoter fused to a β-glucuronidase (GUS) reporter yielded expression in leaves as well as roots of transgenic rice plants. The initial construct used for these experiments used an *ocs* 3′ sequence, and we are presently investigating the possibility that different spatial regulation will be obtained with a construct that bears RCg2 gene 3′ sequence. Another intriguing finding that remains to be explained is that, although the RCg2 reporter constructs (with either the *ocs* or RCg2 3′ end) yield strong transient expression after biolistic (Sanford 1990) introduction into tobacco leaves, no expression has been detected in tobacco stably transformed with these constructs (Xu *et al.* 1994).

Although the findings described above raise questions regarding spatial regulation by rice gene promoters, it is evident that rice promoters do drive cell-specific expression in transgenic plants. Figure 2 shows the interesting contrast between expression of GUS from the RCg2 gene promoter and that from the cyclophilin 1 (*Cyp1*) promoter. Expression from the RCg2 promoter occurs in differentiated zones of the transgenic rice root, and in the root cap, but not in meristematic areas. Conversely, expression from a chimeric construct containing the *Cyp1* promoter fused to GUS (Buchholz & Hall 1994) is intense in meristematic and early differentiating tissues. Expression from the cyclophilin promoter was expected in such tissues because this protein has been shown to exhibit peptidyl-prolyl *cis-trans* isomerase activity (Fischer *et al.* 1989; Takahashi *et al.* 1989) and it probably assists in the correct folding of proteins during synthesis.

The discrete and differential expression patterns in various zones of rice roots seen in figure 2 demonstrate the feasibility of accurately targeting expression of novel traits introduced by genetic engineering to specific cells. In addition, the loss of expression of GUS driven by the *Cyp1* promoter as the root cells differentiate provides confirmation that expression of the introduced trait can indeed be turned off. These examples of spatial and developmental controls show that, for example, a powerful nematocide expressed from an appropriate promoter can be highly expressed in some cells and completely absent from others, such as seed and seed products.

Interestingly, the rice genome contains only a single copy of the gene encoding cytosolic triosephosphate isomerase (TPI) (Xu & Hall 1993; Xu *et al.* 1993). This enzyme catalyses the interconversion between hydroxyacetone phosphate and glyceraldehyde-3-phosphate, an essential step in glycolysis. Surprisingly, although a chimeric TPI promoter-GUS construct yielded substantial transient expression in tobacco leaf tissues, no convincing evidence for transient expression (from biolistic experiments) has been obtained in rice leaves and transgenic rice plants containing this construct show no GUS activity. As in the case of the RCg2 construct, the 3′ sequence in the initial constructs tested was the *ocs* sequence. It will be instructive to determine the activity of constructs bearing a 3′ TPI sequence.

5. ENGINEERING DESIRABLE AGRONOMIC TRAITS INTO RICE

Dramatic advances in crop improvement have been made over the past century through the application of genetics and plant breeding. Genetic engineering provides many new opportunities for crop improvement (Hall *et al.* 1990; Kung & Wu 1993) that are not constrained by sexual compatibility and novel approaches for controlling or eradicating diseases resulting from insect pests or viruses are especially exciting. Rice water weevils (*Lissorhoptrus oryzophilus*) feed briefly on rice seedlings as they emerge through the water and lay eggs in submerged leaf sheathes or culms. Larvae, which cause the most damage to the plant, hatch in about a week and migrate to the rhizosphere. Four weeks of larval feeding severely prunes the root system, resulting in stunted growth, delayed maturity and reduced yield. Crop losses resulting from rice water weevil infestations are expected to mount in the United States since environmental concerns have led to a ban on granular carbofuran, the only effective chemical insecticide available. Additionally, the occurrence of water weevil damage is increasing in Asia (Shih 1991). The expression of an insecticide within the rice plant is a very attractive alternative to the external application of chemical agents. Several proteins are known to have potent insecticidal properties, and we hope to express a protein that is lethal to the rice water weevil. Ultimately, we want to express the protein only in those plant tissues that are attacked by the rice water weevil and only during those developmental periods at which the plant is susceptible. This will assist in public acceptance of genetically modified rice and also limit the energy expended by the plant in producing a novel protein. Consequently, our basic studies on the spatial expression of promoters active in rice tissues are

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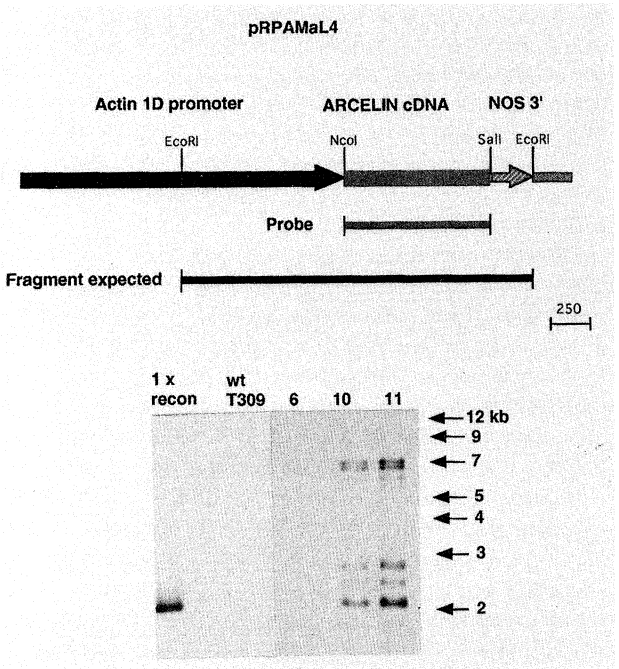

Figure 3. Diagram of the arcelin construct pRPAMaL4 of potential use against the rice water weevil. The upper panel shows the gene construct, the probe used in the Southern blot and the fragment expected to be integrated. The scale bar indicates 250 b.p.. The Southern blot in the lower panel shows a single copy reconstruction of the expected arcelin fragment, the lack of signal from wild-type Taipei 309 probed for arcelin and results for three plants (6, 10 and 11). Plant 6 is negative; plants 10 and 11 are positive but the similar integration pattern shows that they are derived from the same transformed protoplast. Their genome contains a single copy of the intact gene, plus several rearranged copies present in the larger fragments.

cogent to the long-term practical goal of improvement of this crop through biotechnological approaches.

Arcelin is a protein found in the seeds of a limited number of bean (*Phaseolus vulgaris*) cultivars, and evidence has been presented (Schoonhoven *et al.* 1983; Osborn *et al.* 1988) for its toxicity to the bean weevil (*Acanthoscelides obtectus*) and the Mexican bean weevil (*Zabrotes subfasciatus*), both of which are in the same family as the rice water weevil. Corresponding cDNA and genomic clones to arcelin have been isolated and sequenced (Anthony *et al.* 1991) and the arcelin coding sequence has been placed under the control of actin and other promoters (figure 3). These constructs have been introduced into transgenic rice and a plant for which Southern evidence (figure 3) has been obtained for the integration of an intact copy of the actin–arcelin construct is currently under evaluation for its ability to resist infestation by the rice water weevil.

Modified versions of the *Bacillus thuringiensis* (B.t.) crystal toxin (Sutton *et al.* 1991) and various protease inhibitors (Thornburg *et al.* 1987; Wingate *et al.* 1989) are among other attractive candidates for protein insecticides for the rice water weevil, and genes encoding these proteins have been placed behind promoters active in rice (Buchholz *et al.* 1994). We hope that, by the time an active insecticidal compound is obtained, we will have identified monocot promoters providing the desired spatial and temporal regulation of expression.

Viral diseases of rice cause enormous crop loss each year. We are collaborating with research groups at the John Innes Institute (J. W. Davies and R. Hull) and at the University of Nottingham (E. C. Cocking, M. R. Davey and P. Lynch) to develop rice resistant to the rice tungro virus complex. Initial efforts are focused on the coat protein cross-protection strategy (Beachy *et al.* 1990), but other approaches are also under development. Plants containing a construct designed to express a rice tungro spherical virus coat protein coding fragment supplied by the John Innes group have been produced and are presently being evaluated for resistance.

Strategies to be explored for conferral of viral resistance to rice will also include the expression of antisense RNAs to regions that serve as subgenomic promoters. These will be modeled on gene sequences that have proven to reduce brome mosaic virus replication in barley protoplasts (Huntley & Hall 1993). Additionally, expression of defective-interfering (DI)-RNAs (Marsh *et al.* 1991*a*,*b*) in transgenic rice is being pursued as another novel approach against virus infection.

6. DISCUSSION

We are presently in the early stages of development and testing of transgenic rice plants containing novel genes capable of conferring beneficial agronomic traits. Major barriers, such as unreliable procedures for regeneration, have been overcome in some instances, but many still remain. These include the difficulty experienced in combining transformation and regeneration for elite cultivars and the relative infrequency with which viable seed is obtained from transgenic plants. While substantial progress is being made to address these problems, procedures for direct integration of foreign DNA sequences currently yield unpredictable numbers of gene inserts, frequently bearing many rearrangements and deletions. Although this short article has focused on results using the electroporation approach, problems of high gene copy number and rearrangement attend all methods for direct introduction of naked DNA into the genome. In addition to such difficulties, the biolistic approach (Sanford 1990) requires special selection against the regeneration of plants consisting of a chimeric array of cells, some of which are transformed with the GOI, others not.

Researchers must be careful to develop and report rigorous testing of transgenic plants. Definitive data for the presence of the GOI, together with the desired 5′ and 3′ regulatory regions, are all too often lacking from publications claiming successful transformation. Even when the entire gene is present by biochemical analysis, sound evidence for its functionality should also be provided. In this way, the exciting and tantalizing goals for dramatic improvement of crops

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through genetic engineering will steadily but surely be attained.

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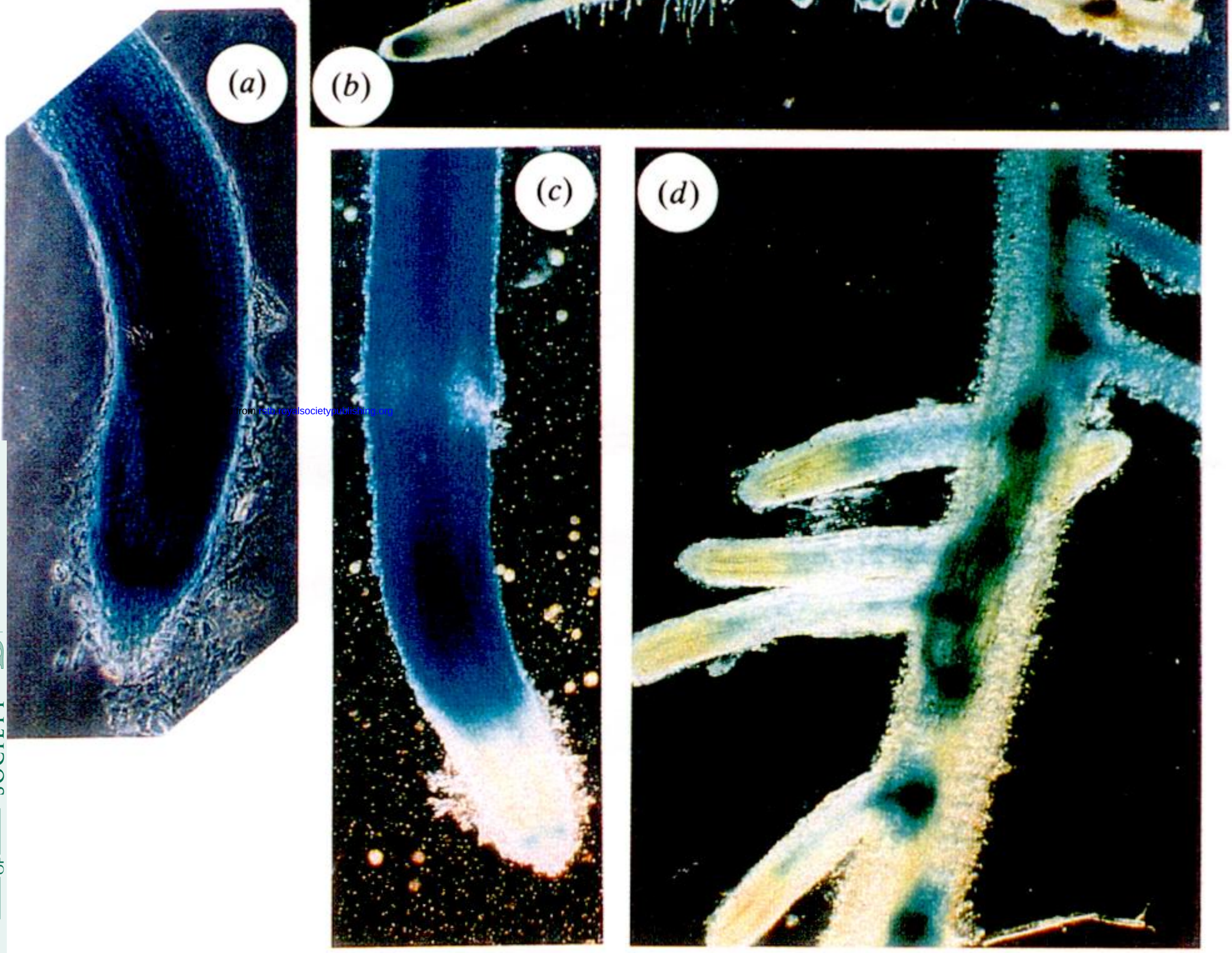

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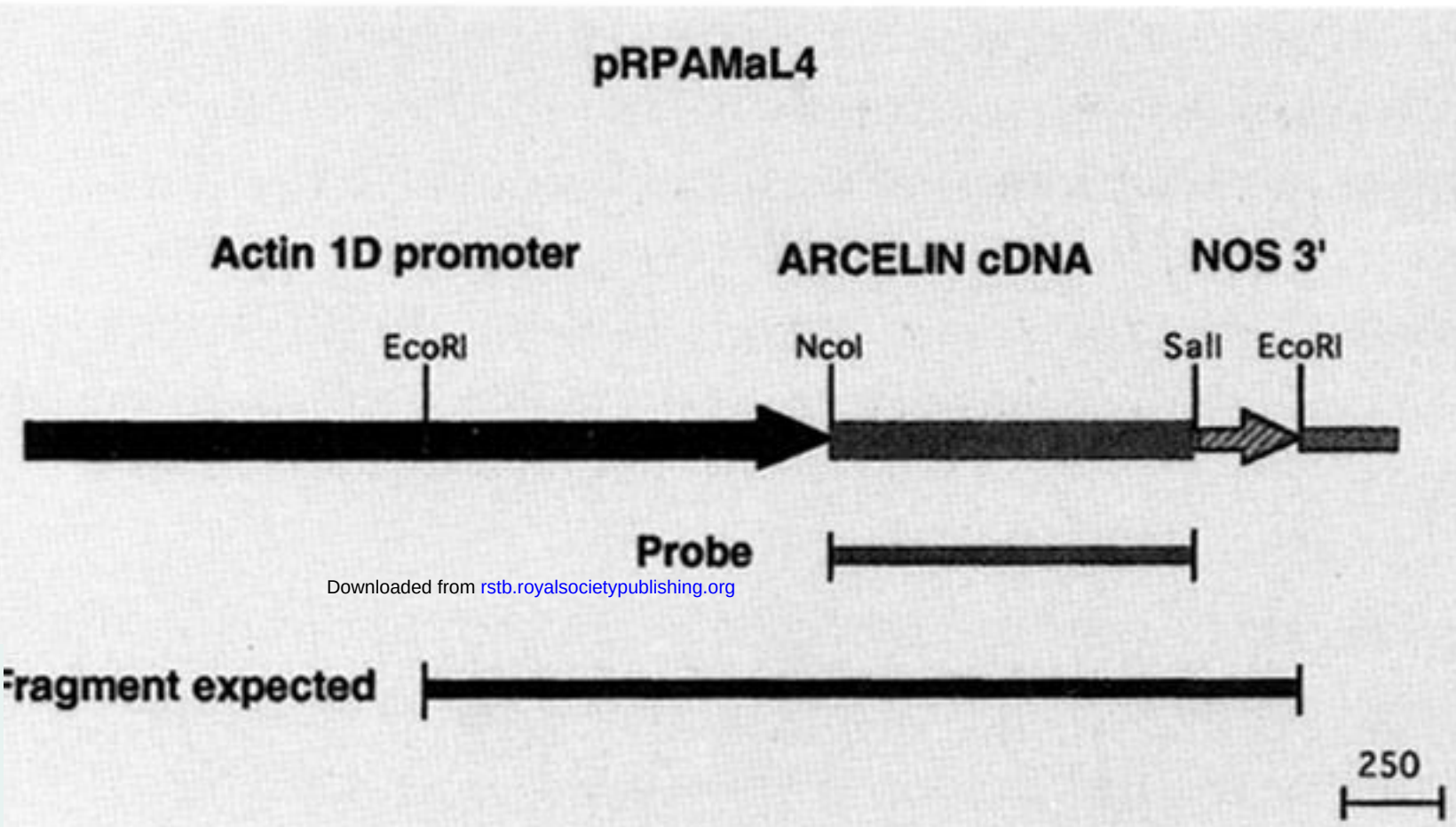

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