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Technical Brief

A heterologous expression system for bovine lens transmembrane Main Intrinsic Protein (MIP) in *Nicotiana tabacum* plants

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We have developed a heterologous expression system for transmembrane lens main intrinsic protein (MIP) in *Nicotiana tabacum* plant tissue. A native bovine MIP26 amplicon was subcloned into an expression cassette under the control of a constitutive Cauliflower Mosaic Virus promoter, also containing a neomycin phosphotransferase operon. This cassette was transformed into *Agrobacterium tumefaciens* by triparental mating and used to infect plant tissue grown in culture. Recombinant plants were selected by their ability to grow and root on kanamycin-containing media. The presence of MIP in the plant tissues was confirmed by PCR, RT-PCR and immunohistochemistry. A number of benefits of this system for the study of MIP will be discussed, and also its application as a tool for the study of heterologously expressed proteins in general.

Alterations to the structure and function of the vertebrate lens transmembrane 26 Kd main intrinsic protein (MIP) have been implicated in the development of cataract, the leading cause of blindness in the world human population. MIP accounts for over 60% of lens fiber cell membrane intrinsic protein. Hypotheses for its function have included gap junction, adhesin-like and channel roles, and as a nexus for interaction with other lens proteins such as crystallins. Moreover, MIP is a representative of an important family of membrane proteins, which includes the aquaporins, and which has been largely conserved throughout evolution [1].

However, a firm elucidation of MIP function remains recalcitrant. MIP is only found in the final stages of lens fiber cell differentiation. Mature fiber cells, which make up the great bulk of the lens no longer contain organelles and have no aerobic respirative or genetic activity. Therefore, they cannot be cultured in vitro and so MIP cannot be studied in its main cellular environment. Therefore, a variety of heterologous expression systems have been developed for the study of MIP. To date, MIP has been expressed in *E. coli* [2], *Xenopus* oocytes [3], *Spodoptera frugiperda* (SF) cells [4,5], and recently mammalian erythroid leukemia (melC) cell lines (Drake and Crabbe, unpublished).

However, a number of characteristics of the heterologously expressed MIP differ between the different systems: MIP is non-glycosylated in *E. coli* and *Xenopus* oocytes, but significantly glycosylated in SF cells. MIP may not be phosphorylated in SF21 cells (de Peyer and Crabbe, unpublished), even though phosphorylation of MIP is known to be an important regulatory mechanism in vivo. MIP appears to be a weak glycerol channel in *Xenopus* oocytes [6], but not in *E. coli* (de Peyer and Crabbe, unpublished). *E. coli* studies indicate a 7 transmembrane region topology, whilst SF21 cells and MelC cells indicate 6 transmembrane topologies. Clearly, additional heterologous expression systems need to be developed so that a consensus can be reached for the structure and function of MIP.

Heterologous expression of mammalian proteins in plant tissue is a promising candidate since expression is stable and permanent (not transient as in *E. coli* or insect cells) and shows post-translational modifications similar to mammalian tissues. Transgenic plants have already been successfully engineered to heterologously express members of the MIP family from other plant species [7]. We have now extended this to mammalian lens MIP itself.

METHODS

Expression constructs: A bovine MIP amplicon bound by a 5' *Kpn I* restriction endonuclease site and a 3' *Sma I* site was produced by PCR using appropriate primers. This was ligated into the relevant sites of an expression cassette consisting of the bovine MIP cDNA under the control of a constitutive Cauliflower Mosaic Virus promoter [8] and a kanamycin resistance operon [8], flanked by Left and Right Border sequences from the *Agrobacterium tumefaciens* Ti plasmid [8]. This cassette can be seen in Figure 1.

Sequence of MIP N-terminal primer: The dinucleotide marked with green is a nonsense sequence. The sequence marked in red is the engineered *Kpn I* sequence. The slash indicates the restriction enzyme cut site. The rest of the sequence codes for a generic MIP N-terminal sequence.

5'-ACGGTAC/CATGTGGGAACTGCGGTCAGCC-3'

Sequence of MIP C-terminal primer: The dinucleotide marked with green is a nonsense sequence. The sequence

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marked in red is the engineered *Sma I* sequence. The slash indicates the restriction enzyme cut site. The rest of the sequence codes for a generic MIP C-terminal sequence.

5'-ACCCC/GGGTTACAGGGCCTGGGTCTT-3'

PCR cycle parameters: Melting step: 94 °C for 1.5 min, annealing step: 60 °C for 2 min, elongation step: 72 °C for 2 min. At termination of 30 cycles, there was a further, final elongation step of 72 °C for 10 min.

Transformation: Transformation of *A. tumefaciens* and thence plant tissue with this expression cassette was essentially as per reference [8]. The expression cassette was cloned into the disabled *A. tumefaciens*LBA4404 strain by triparental mating (Helper plasmid pRK2013; Helper strain JM101). The resultant recombinant strain was used to infect *Nicotiana tabacum* (cultivar Wisconsin) leaf disks. Transgenic disks produced calli on kanamycin (250 µg/ml) selective medium. Shoots from calli were excised and transferred to fresh selective medium.

RNA and DNA extraction and amplification: Plant genomic DNA was extracted using the Qiagen(Crawley, West Sussex, UK) "DNAeasy" kit and accompanying proprietary protocol (Qiagen, catalogue number 69103). Plant mRNA was extracted using the Dynal (Bromborough, Wirral, Meseyside, UK) Dynabeads mRNA DIRECT kit and accompanying proprietary protocol (Dynal, catalogue number 610.11). Plant genomic DNA, and cDNA generated from plant mRNA after reverse transcription, were both used as templates in PCR with the primers described above, followed by agarose gel electrophoresis analysis [9]. Reverse transcription was carried out using the Promega (Southampton, UK) Access RT-PCR kit and accompanying proprietary protocol (Promega, catalogue number A1250).

Immunohistochemistry: Immunohistochemical analysis of tissue samples from two different plant organs (young leaves, and roots) was carried out according to a proprietary protocol developed at Oxford Brookes University, UK (This protocol proved unsuitable for other tissues).

Examination of proteins in plant cells is complicated, since even minor disruption of plant tissues releases vacuolar pro-

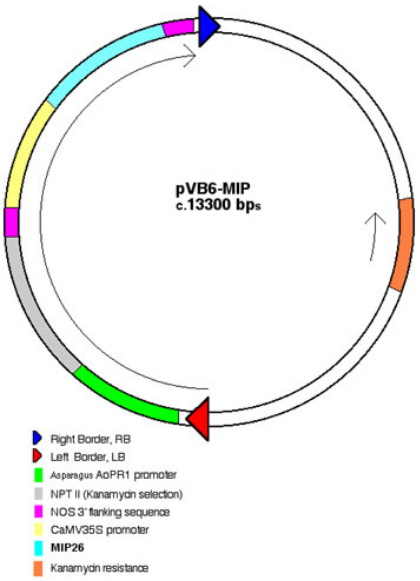

Figure 1. Cloning strategy for ligation of MIP26 into pSIN and thence into pVB6. Both MIP and the kanamycin resistance factor Neomycin phosphotransferase (NPTII) are flanked at their 3' end by Nopamine Synthase (NOS) flanking sequence [8], part of the wild-type *A. tumefaciens* Ti plasmid [8]. Genes appended with this sequence have been demonstrated to be transferred to plant tissue more efficiently [8]. Since MIP receives its own Cauliflower Mosaic Virus (CMV) promoter from the pSIN vector, it is does not matter in which direction it ligates into the final pVB6 vector.

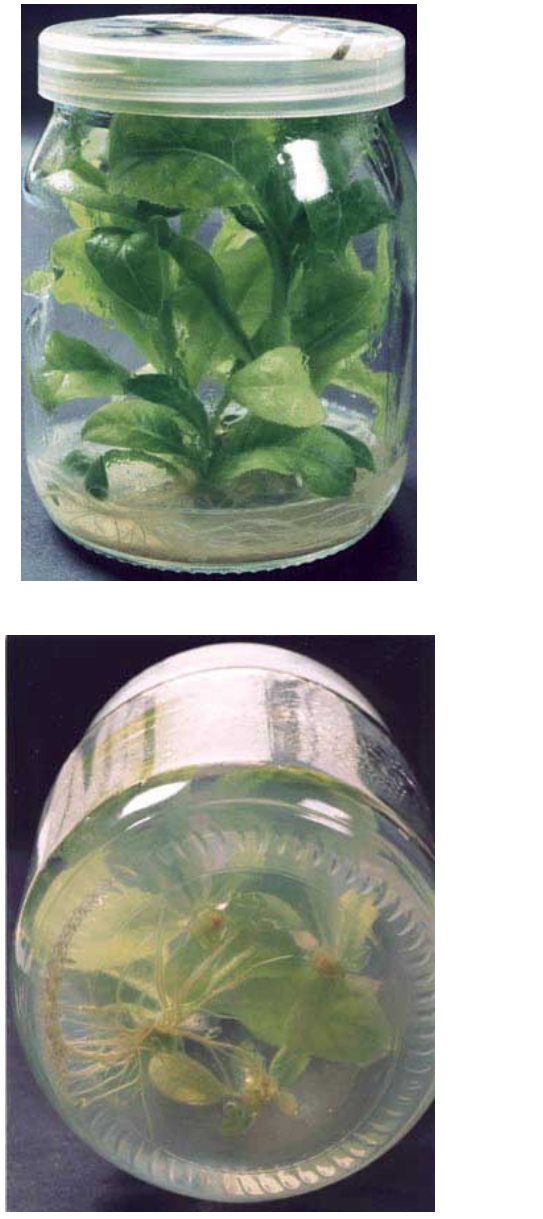

Figure 2. Transgenic plant morphology. **A**. The expression cassette shown in Figure 1 was cloned into the disabled *A. tumefaciens* LBA4404 strain by triparental mating. The resultant recombinant strain was used to infect *Nicotiana tabacum* leaf disks. Transgenic disks produced calli on kanamycin-selective medium. Shoots from calli were excised and transferred to fresh selective medium and allowed to develop into full plants as shown here. **B**. Detail of roots. Note rooted and non-rooted phenotypes; the latter is non-recombinant.

teases which rapidly degrade cellular proteins. Therefore, no sample could be obtained for simple analyses such as SDS-PAGE or Western blotting. Instead, samples were explored immunohistochemically, after fixing and sectioning in Butyl-Methyl Methacrylate resin, according to the following protocol:

Fixation: Roots and young leaves were dissected, and were fixed in 4% paraformaldehyde in 50 mM Pipes (Piperazine-N,N'bis[2-ethanesulfonic acid]) buffer plus 1 mM $CaCl_2$, pH 6.9 for 2 h at room temperature, followed by 3 washes of 10 min each in 50 mM Pipes plus 1 mM DTT (pH 6.9). Root samples were then dyed with Fast Green to make them easier to see in subsequent manipulations.

Dehydration: Samples were dehydrated by transfer using tweezers to the following solutions. Each step had a duration of 30 min at the temperature indicated:

10% Ethanol + 1 mM DTT, 4 °C, rotated on "windmill"
25% Ethanol + 1 mM DTT, 4 °C, rotated on "windmill"
50% Ethanol + 1 mM DTT, 4 °C, rotated on "windmill"
70% Ethanol + 1 mM DTT in cryostat at -20 °C
90% Ethanol + 1 mM DTT in cryostat at -20 °C
100% Ethanol + 1 mM DTT in cryostat at -20 °C

Infiltration: Butyl-Methyl Methacrylate resin was then infiltrated into the samples by transfer using tweezers into the following solutions, all at -20 °C:

2:1 Ethanol:Resin + 1 mM DTT overnight
1:1 Ethanol:Resin + 1 mM DTT 2 h
1:2 Ethanol:Resin + 1 mM DTT 2 h
100% Resin + 10 mM DTT, 3 h or overnight

Polymerization: The infiltrated samples were then degassed with dry Nitrogen gas for 30 min. Samples were then transferred to aluminium dishes of fresh resin and polymerized at 0 °C under UV light (360 nm) overnight. A Nitrogen atmosphere (gas flow of 450 ml/min) was maintained throughout the polymerization. The resulting blocks of polymerized resin were then stored with dessication until sectioning.

Sectioning and mounting: The methacrylate resin-encased sample blocks were sectioned on a glass knife microtome at 2-4 µm. Individual sections were then transferred to drops of water on wells of multiwell Polylysine-coated slides, and allowed to dry at 37 °C overnight.

Washing, application of antibodies, and examination: The resin was dissolved by standing the slides in acetone for 5-10 min, without agitation. The samples on the slides were then rehydrated by standing in PBST (phosphate buffered saline [PBS] plus 0.05% Tween 20) for 10 min and blocked for 15 min in PBSBT (PBS plus 0.1% Tween 20, 1% BSA, and 0.002% Sodium Azide). Slides were then dried between the wells, and primary anti-MIP antibody was added to each well at the appropriate dilution in PBS. These slides were placed in

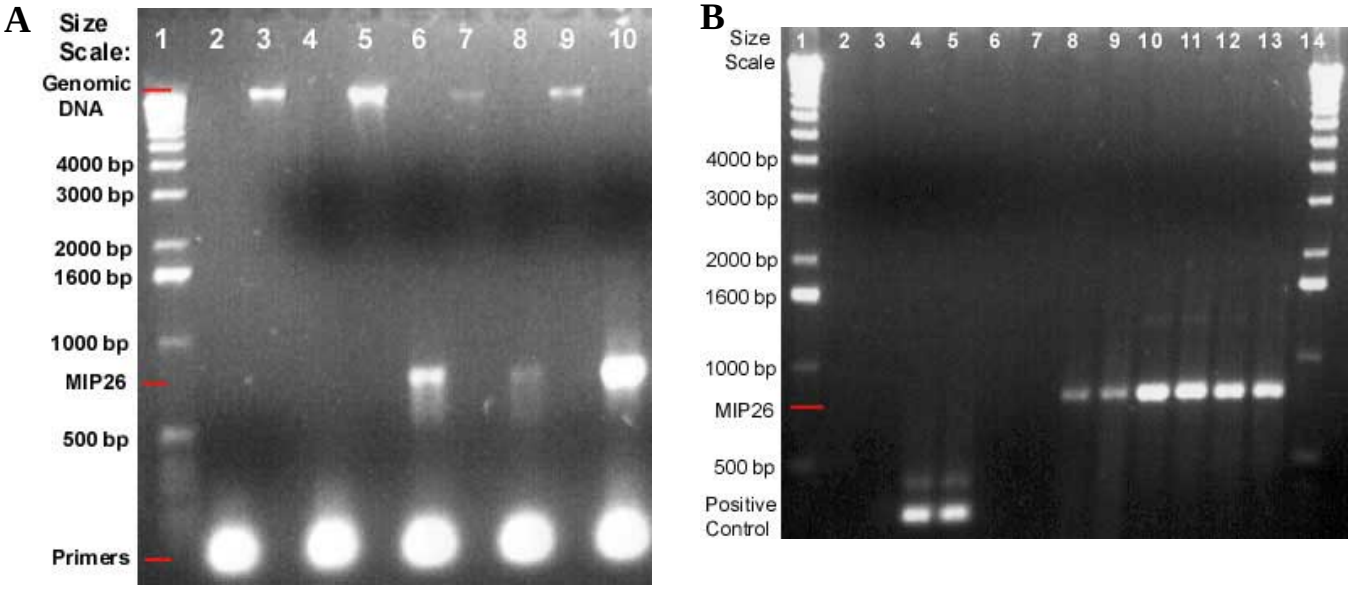

Figure 3. Analysis of transgenic plants for the presence of MIP using PCR and RT PCR. **A**. MIP26 PCR product from plant genomic DNA. Plant genomic DNA was extracted using the Qiagen "DNAeasy" kit. Plant genomic DNA was used as the template in PCR, followed by agarose gel electrophoresis analysis. Lane 1, DNA size markers; lane 2, PCR without template; lane 3, wild-type plant DNA alone (these lanes show that PCR contamination precautions were adequate to prevent aerosol contamination and false positive bands); lane 4, lack of PCR products from wild-type plant DNA; lanes 5, 7 and 9, DNA from transgenic plants; lanes 6, 8, and 10, PCR products from DNA from transgenic plants. Lanes 6, 8, and 10 contained a band of about 800 bp in size, which was excised, sequenced, and confirmed as bovine MIP26. **B**. MIP26 RT PCR product from plant mRNA. Plant mRNA was extracted using the Dynal Dynabeads mRNA DIRECT kit. Plant cDNA generated from plant mRNA after reverse transcription was both used as a templates in PCR, followed by agarose gel electrophoresis analysis. Reverse transcription was carried out using the Promega Access RT-PCR kit. Lanes 1 and 14, DNA size markers; lanes 2 and 3, RT-PCR with no template; lanes 4 and 5, positive control RT-PCR with known mRNA; lanes 6 and 7, wild-type plant mRNA (these lanes show that the primers employed were be specific enough to not inadvertently amplify native plant cDNAs); lanes 8, 9,10,11,12 and 13, mRNA from tansgenic plants. These reactions gave a band of about 800 bp in size, which was excised and sequenced, and confirmed as bovine MIP26. The positive control (lanes 4 and 5) was as supplied with the Promega Access RT-PCR kit (mRNA for the E. coli kanamycin resistance gene), giving rise to a 323 bp amplimer using the primers supplied with the kit.

a moist sealed container at 4 °C overnight, followed by incubation at room temperature for 2 h the following day. The slides were then washed in PBST three times for 10 min each. The PBST was individually pipetted and removed from each well. Secondary antibody (anti-Rabbit FITC [Fluoroisothiocyanate] conjugate) was then added at 1:40 dilution for 2 h at room temperature. The wells were then washed in PBS three times for 10 min each. Propidium iodide nuclear stain (3 µg/ml) was then briefly applied to each well followed by two washes in PBS and one wash with water. Coverslips were then mounted on the slides with Citifluor antifadeant and sealed with nail varnish. Slides could be examined immediately using fluorescence microscopy or stored at 4 °C.

RESULTS

Putative transgenic plants showed consistent root growth on kanamycin selective medium. Rooting is an excellent indicator of the presence of the expression cassette in plant tissue (Figure 2A,B). Beyond the ability to root in kanamycin selective medium, there was no difference in phenotype between transgenic plants and control plants grown on non-selective medium.

Rooted plants screened by PCR and RT-PCR exhibited a single PCR product of ~800 nucleotides (Figure 3A) which sequencing confirmed as bovine MIP. The presence of this band from mRNA (Figure 3B) confirms transcription of the cassette (mRNA yields from the plants were low, ~10 ng/µl, precluding quantitation). Control plants exhibited neither root growth nor PCR product.

Cell membranes from young transgenic leaves exhibited significantly enhanced immunofluorescence (Figure 4A,B) versus control plants (Figure 4C). Since immunofluorescence is localized to cell membranes, this suggests that MIP has been correctly targetted to young transgenic leaf cells within the plants.

DISCUSSION

We have succeeded in generating transgenic plants expressing bovine lens MIP. The phenotype of the plants did not differ from control plants, in contrast to a previous example of heterologous expression of an MIP family member [7] in *N. tabacum* where morphology was significantly affected.

We observed one sample of increased immunofluorescence in a young root. It is possible that MIP is only expressed in juvenile transgenic roots, although this would be hard to explore further since juvenile specimens would be very close to the main mass of roots and so hard to excise (only the outermost root hairs can be conveniently dissected away for analysis). Likewise, the distribution of MIP protein in other transgenic plant tissues is not known at present, due to their unsuitability for immunohistochemistry.

Currently, it is not possible to usefully quantify levels of transcription and translation of the MIP transgene. Recombinant genes in transgenic plants are not necessarily stable from one generation to the next. For instance, the introduced gene might lie within a methylation island in the plant genome, and become itself methylated and inactive with continued sub-culturing of the plant line. For this reason, in ongoing work the transgenic plant lines expressing MIP26 are being grown in a glasshouse environment to sexual maturity. Backcrossing of plant lines should result in a stable plant line; also the level of MIP expression can be maximized by crossbreeding different high-expressing lines.

This expression system has potential in the elucidation of MIP structure and function. Protoplasts can be isolated from plant tissues [10] and subjected to electrophysiological analy-

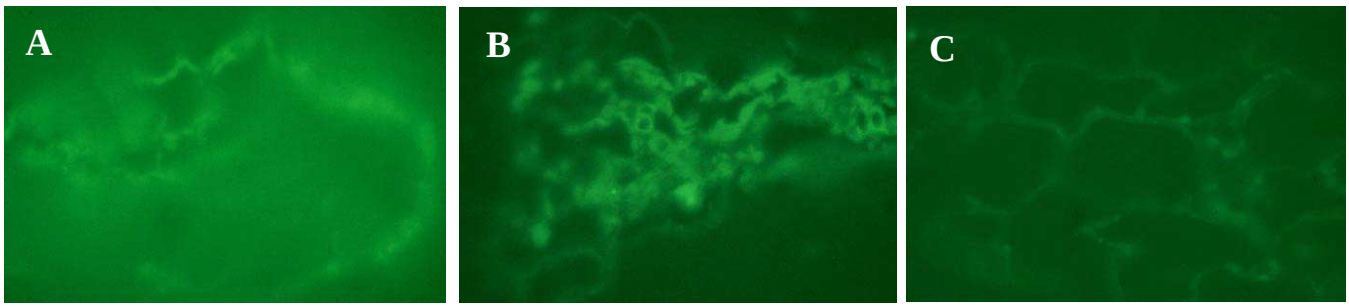

Figure 4. Immunohistochemical analysis of young leaf and root tissues. Some slides showed irregular cell structure since adhesion of the microtomed samples to the slides was poor; they had a tendency to lift off partially and present a distorted or corrugated appearance. This is an inherent drawback of plant tissues and we are aware of no superior plant immunohistochemical protocol for light microscopy. The magnification was x200 for all figures. The longest dimension of each image is approximately 180 µm. All our controls, including those with primary and secondary antibodies alone, were negative and showed no fluorescence. **A**. Young transgenic leaf section showing enhanced immunofluorescence at cell peripheries. Young leaves were dissected, and fixed in 4% paraformaldehyde. Slides were dried, and primary anti-MIP antibody was added. Secondary antibody (anti-Rabbit FITC conjugate) was then added. Propidium iodide nuclear stain (3 µg/ml) was then briefly applied, coverslips were mounted on the slides and sealed with nail varnish. **B**. Young transgenic leaf section showing enhanced immunofluorescence at cell peripheries. Young leaves were dissected, and fixed in 4% paraformaldehyde. Slides were dried, and primary anti-MIP antibody was added. Secondary antibody (anti-Rabbit FITC conjugate) was then added. Propidium iodide nuclear stain (3 µg/ml) was then briefly applied, coverslips were mounted on the slides and sealed with nail varnish. **C**. Young leaf section from control plant showing background immunofluorescence. Young leaves were dissected, and fixed in 4% paraformaldehyde. Slides were dried, and primary anti-MIP antibody was added. Secondary antibody (anti-Rabbit FITC conjugate) was then added. Propidium iodide nuclear stain (3 µg/ml) was then briefly applied, coverslips were mounted on the slides and sealed with nail varnish. From our numerous plant (and indeed root) cell sections, we have always seen some background immunofluorescence localized to cell membranes.

sis such as patch clamping [11,12]. Transmembrane topology can be immunolocalized using antibodies raised to discrete peptide sequences from the MIP26 polypeptide. However, immunolocalization of cytoplasmically exposed domains of MIP has been carried out in *E. coli* and SF21 cells through inverting spheroplasts or disruption of cell membranes with agents such as acetone to allow antibodies access to the cytoplasmic side of the membrane [2,4]. These techniques are not applicable to plant cells due to the presence of a cell wall. Therefore, techniques such as gold-labelled antibodies used in conjunction with transmission electron microscopy may be required.

Although low resolution structures have been proposed for some MIP family members through cryoelectron microscopy studies [13-16], this has not been sufficient to distinguish between the different topological models proposed. Moreover, heterologous expression systems developed so far have not provided enough correctly folded recombinant protein to permit crystallisation trials. By contrast, a *N. tabacum* system can effectively be scaled up agriculturally and is not limited by available fermenter technology. Purification of proteins from plant cells is problematic due to vacuolar proteases; to counter this, we have constructed an N terminal oligohistidine-tagged MIP clone for the purposes of Nickel-chelating affinity chromatography, which should aid in the optimisation of MIP26 recovery from a variety of tissues based on existing *N. tabacum*membrane preparation protocols [7].

Plant systems have previously been used for the heterologous expression of a variety of substances on an industrial scale [17-19], including the production of recombinant light-harvesting complexes for crystallisation [20]; here we promote the use of transgenic plants on a laboratory scale, with an inherent and considerable potential for scale-up.

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