MATERIALS AND METHODS

The present study was carried out at the Council of Scientific and Industrial Research-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, H.P., India (32º6’ N latitude; 76º33’ E longitude; 1289 m above mean sea level; amsl) in the Western Himalayas. The materials and methods (M & M) used for raising transgenic potato plants and their evaluation under in vitro and in vivo conditions have been described under the following headings.

3.1. Materials Used in the Present Study

3.1.1. Plant System

Potato (Solanum tuberosum ssp. tuberosum L.), cultivar (cv.) Kufri Giriraj (KG) a member of family Solanaceae, order Solanales of the angiospermic plants was selected for the present study. The general description of cv. KG, which was released in the year 1998 and procured from CPRI, Shimla is listed below (Source: CPRI, Shimla, H.P., India):

3.1.1.1. Parentage

SLB/J-132 (I) x EX/A 680-16 (I)

3.1.1.2. Morphological Characteristics

![Images of potato plant parts]

Fig. 3.1: Morphological characteristics of potato cv. Kufri Giriraj

**Plant:** Plants medium tall, semi-erect, medium compact and vigorous. Stems many, medium thick, colored at base with moderately developed straight wings

**Foliage:** Green

**Leaves:** Open, rachis green, leaflets ovate, smooth dull surface with entire margin
**Flowers**: Light purple, shy flowering. Anthers are yellow, well developed, low pollen stainability and stigma is round

**Tubers**: White, medium to large, oval, smooth skin, fleet eyes and white flesh

**Sprouts**: Light Purple

### 3.1.1.3. Agricultural Characteristics

**Adaptability**: North and South Indian hills  
**Maturity**: Medium (130-135 days)  
**Yield**: 25 tonnes ha\(^{-1}\)  
**Dormancy**: Medium (8-9 wks)  
**Diseases**: Both foliage and tubers are resistant to late blight  
**Dry matter**: Medium

### 3.1.1.4. Consumer and Processing Quality

The tubers of KG are easy to cook, waxy texture, mild flavour and free from discoloration after cooking. However, the tubers are generally not suitable for processing.

### 3.1.2. Genes Employed for Transgenic Development

Thaumatin-like protein (*TLP*) and Polyphenol oxidase (*PPO*) genes were isolated from the *Camellia sinensis* (L.) O. Kuntze at CSIR-Institute of Himalayan Bioresource Technology, Palampur, H.P., India. Using these genes, transgenic potato plants were developed to functionally validate the role of these genes in imparting stress tolerance.

#### 3.1.2.1. Thaumatin-Like Protein (*TLP*) Gene

Thaumatin-like protein gene (*CsTLP*) (Gene Bank sequence accession number **DQ444296.1**; 681 bp) has been isolated from the high altitude plant; *Camellia sinensis* (L.) O. Kuntze cv. Teenali, which grows in the experimental tea farm of Institute of Himalayan Bioresource Technology, Palampur. The plant belongs to the family *Theaceae* whose leaves and leaf buds are used to produce the popular beverage tea. Muoki et al. (2012) recorded an early up-regulation of *CsTLP* during different environmental stresses in *Camellia sinensis* cultivar UPASI-9.

#### 3.1.2.2. Polyphenol Oxidase Gene

*Polyphenol oxidase* (*PPO*) (Gene Bank sequence accession number **FJ656220.1**; 1800 bp) has been isolated from the plant; *C. sinensis* (L.) O. Kuntze which grows in the
experimental tea farm of the Institute of Himalayan Bioresource Technology (32°6’ N latitude; 76°33’ E longitude; 1289 m amsl), was selected and characterized at IHBT-CSIR, Palampur. PPO is known to catalyze the oxidation of phenolics to quinines, which are toxic to pathogens (Meyer and Harel 1979). The free radicals of quinine can react with biological entities, thus creating unfavourable environment for pathogen development (Duffy and Felton 1991). The inactivation of pathogen’s proteolytic enzymes by the oxidized substrate of PPO is reported in various host pathogen combinations and considered as a part of the defense mechanism.

3.1.3. Vector Used

For raising transgenic potato plants the C. sinensis TLP (CsTLP) and C. sinensis PPO (CsPPO) genes were separately cloned in the binary vector pCAMBIA1302 (10,549 bp) having kanamycin resistance gene (nptII) as bacterial selection marker and hygromycin resistance gene (hpt) as a plant selection marker. The fusion protein as Green Fluorescent Protein (GFP) is present as the reporter gene for in situ expression studies in plants. The CsTLP was cloned between NcoI and SpeI restriction sites whereas CsPPO was cloned in between NcoI and SpeI restriction sites. Both the genes and the green fluorescent protein (GFP) are driven by the constitutive promoter CaMV35S and terminated by nopaline synthase polyA terminator. The bacterial and plant selection antibiotic resistance genes are also driven by the constitutive promoter, CaMV35S (Fig. 3.2).
3.1.3.1. Bacterial Strains

The pCAMBIA1302 vectors harboring separately CsTLP and CsPPO genes were transformed into E. coli DH5α strain, kindly provided by Dr. Sanjay, Asosii Paul, Dr. Y. Sreenivasulu and Dr. Tejpal Gill, CSIR-IHBT, Palampur. Helper strain pRK2301 in E. coli DH5α was used for transferring the transgenes from E. coli to Agrobacterium tumefaciens strain GV3101 (Koncz and Schell 1986), which was used for further transformation studies. GV3101 is resistant to rifampicin, sensitive to kanamycin and compatible with binary vector pCAMBIA1302.

3.1.4. Primer Designing

3.1.4.1. Design of PCR and Expression Primers

The coding sequences of CsTLP and CsPPO genes were collected from the NCBI site (Appendix III). Primers were designed using Primer 3 Software (http://www.genome.wi.Mit.Edu/cgi-bin/primer/primer3.Cgi) for PCR confirmation of CsTLP and CsPPO in the transformed Agrobacterium strains by colony PCR, integration and expression studies of CsTLP and CsPPO in transgenic plants (Table 3.1).

The amplification of ITS 1, 5.8S ribosomal RNA gene and ITS 2 was achieved using ITS 1 (forward primer) and ITS 4 (reverse primer) (White et al. 1990). ITS 1 and ITS 4 primers designed to amplify rDNA for fungal characterization are shown in Table 3.1.

3.1.4.2. Design of quantitative Real Time-PCR (qRT-PCR) Primers

qRT-PCR primers were designed from the coding sequences of potato defense response pathway genes viz. Thaumatin-like protein (StTLP), lipoxygenase (StLOX) and Phenylalanine ammonia lyase (StPAL) genes downloaded directly from NCBI database. Primers were designed using Primer 3 Software (http://www.genome.wi.Mit.Edu/cgi-bin/primer/primer3.Cgi). The parameters for designing of primers were kept as default with amplicon length as 100 to 120 bases; primer length, 18-20 bases; Tm 54 to 60°C; percent GC concentration varies from 30 to 80% and there should be less than two G+C with in five nucleotide at 3’ end. List of primers used for qRT-PCR analyses were shown in Table 3.2.
Chapter 3

Materials and Methods

3.1.5. Polymerase Chain Reaction (PCR)

For general PCR and to check the integration and expression of genes in the transgenic plants, genomic DNA and cDNA, respectively, were used as a template in PCR reaction. Components added for 25 µl of PCR reaction mixture were: 80-120 ng of template DNA, 0.2 mM dNTPs mix, 0.4 µM each of forward and reverse primer, 1.2 U of Taq polymerase in 1X Taq buffer (Bangalore Genei, India). The PCR conditions for all the genes are

<table>
<thead>
<tr>
<th>Genes integration and expression studies</th>
<th>Primer</th>
<th>5’-3’ sequences</th>
<th>Amplicon size (bp)</th>
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<td>5’-ATG AGC TTC CCC AAA AGC CT-3’</td>
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<td></td>
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<td>5’-TCA AGG GCA GAA GGT AAT AGC-3’</td>
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</tr>
<tr>
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<td>Forward</td>
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<td></td>
<td>Reverse</td>
<td>5’- GTG ACT ATC TTG GTA ATC-3’</td>
<td>-</td>
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<tr>
<td>CsPPO + Integration</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5’- ATACTAGTAGAAATCAACTCAATTTGAC-3’</td>
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<tr>
<td>CsPPO + Expression</td>
<td>Forward</td>
<td>5’- CAACTCATGGCTTCTTTTCTC-3’</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5’- CGGGTCAGTCAAATGTAGT-3’</td>
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Molecular characterization of fungus

<table>
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<tr>
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<td>Reverse ITS4</td>
<td>5’-GCT GCG TTC ATC GAT GC-3’</td>
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Table 3.2: Specialized primers used for quantitative Real Time PCR

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<tr>
<th>Gene</th>
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<th>Primer sequence (5’-3’)</th>
</tr>
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<td>StLOX (Lipoxygenase)</td>
<td>AF039651</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-AAG GTC CCT GAG GAA GTT TTG A-3’</td>
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<td>StPAL (Phenylalanine ammonia-lyase)</td>
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<td></td>
<td>Reverse</td>
<td>5’-CAC CAG CTC TTG CAC TCT C-3’</td>
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<td>StTLP (Thaumatin like protein)</td>
<td>AY737315</td>
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<td>CsTLP (Camellia sinensis thaumatin like protein)</td>
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<td></td>
<td></td>
<td>Reverse</td>
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| StERS1 | Forward | 5’-TGG TGG GTA CTC CGA TA CAT -3’ |
| | Reverse | 5’-CGG TGG AGG AAA GGT AAG GT-3’ |
summarized in Table 3.3. The amplified product was analyzed on a 1.2% agarose gel using a 100 bp ladder. For specific PCR or any deviation with respect to reaction volume and PCR components were described separately in that section.

3.1.6. Description of Standard in Vitro Culture Conditions and Basal Medium

In general, in vitro culture conditions used in the present research work were: temperature 25 ± 2°C, relative humidity 50-60% and light intensity of 60-70 µmol m⁻² s⁻¹ with 16 h light period. Basal medium used for in vitro culture was Murashige and Skoog (MS; Murashige and Skoog 1962) supplemented with 2% sucrose and pH 5.7 (hereafter referred to as MS basal medium). The media was solidified with 0.8% agar and autoclaved at 121°C for 20 min. Any other culture conditions or medium used has been described separately.

Table 3.3: PCR condition for different primers

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<th>Genes/Assay</th>
<th>Parameters</th>
<th>Initial Denaturation</th>
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Table 3.4: Thermal profile for quantitative Real Time PCR

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3.2. Establishment of Aseptic Shoot Cultures and Micropropagation

3.2.1. Establishment of Aseptic Shoot Cultures

In vitro plants were obtained from Central Potato Research Institute (CPRI), Shimla, India (altitude ~2202 m, amsl; 32°29’ 19” N; 75°10’ 46” E) and maintained in plant growth chambers (Kaleidoscope, Banglore, India) at CSIR-IHBT, Palampur, India. In vitro grown plants were hardened inside the hardening chamber (set at temperature 25 ± 2°C; relative humidity (RH; 70%) for 3 wks and transplanted in poly-houses to raise minitubers. To achieve the objectives set in the present thesis, shoot cultures were raised from minituber sprouts and sub-cultured on MS basal medium (Murashige and Skoog 1962). To remove latent bacteria from the shoot cultures, different concentrations of filter sterilized streptomycin sulfate (0, 50, 100, 250 and 500 mg l⁻¹) were added into MS medium under laminar air flow (LAF) cabinet. The efficacy of antibiotic in removing latent bacteria and phytotoxicity related to its incorporation in MS basal medium was evaluated after a period of two wks. Aseptic shoot cultures were maintained in 250 ml flasks with 100 ml MS basal media and incubated under in vitro culture conditions mentioned earlier (Section 3.1.6).
3.2. Effect of Silver thiosulfate (STS) in reducing ethylene induced culture abnormalities during micropropagation

Single node cuttings (SNCs; with axillary bud and leaf), about 10 to 15 mm in length, of in vitro micro-propagated plantlets were cultured on MS basal medium supplemented with 0, 2, 4, 6, 8, 10 and 12 µM STS. Five flasks each with four SNCs were considered for each treatment. Morphological data including shoot length, internodal length, leaf area, root biomass and leaf biomass were collected after 4 wks of inoculation. Leaf area was calculated by multiplying length and breadth with leaf area index (0.68 for potato cv. KG). Leaf area index for cv. KG was obtained by dividing the actual leaf area marked on a graph sheet by the product of length and breadth of leaf. Biochemical analysis involves, leaf chlorophyll content estimation from leaves of the shoot cultures growing at 0-12 µM STS. For the sake of comparison, leaf chlorophyll content was also estimated from in vivo potato plants. Morphological changes i.e. trichome development and vascularization in response to STS was determined by clearing the leaves followed by microscopic observations.

On the basis of the morphological and biochemical results, shoot cultures grown at 4 µM STS proved to be beneficial in terms of shoot length, internodal length, leaf biomass and leaf area required for regeneration studies and generation of transgenic plants. Therefore, further study to show the involvement of silver ions in regulating the transcript levels of Solanum tuberosum ethylene responsive gene (StERS1) were carried out on plants grown on MS basal medium supplemented with 4 µM STS and compared to those grown on MS basal medium.

3.2.2.1. Preparation of STS Stock Solution

The STS stock solution of 6 mM (1.96 g l⁻¹) was prepared by mixing 2.04 g l⁻¹ of (silver nitrate) AgNO₃ and 23.82 g l⁻¹ of sodium thiosulfate pentahydrate (Na₂S₂O₃·5H₂O) in a 1:1 ratio (Perl et al. 1988). STS stock was freshly prepared, filter-sterilized, stored in the dark at 4°C and was added to the autoclaved media (≈ 45-50°C) in LAF cabinet.

3.2.2.2. Chlorophyll Content

Chlorophyll (Chl a, Chl b and total chlorophyll) was estimated following the method of Arnon (1949). Leaf samples (100 mg) were collected in a 2 ml eppendorf tube with 1 ml 80% (v/v) acetone and crushed using a micro pestle in liquid nitrogen. The leaf extract was vortexed vigorously and kept at -20°C for overnight. Next day the samples were
centrifuged at 10,000 rpm for 15 min and supernatant from each tube was transferred to fresh tube. The pellet was re-suspended in 1 ml of 80% acetone, vortexed and kept at -20°C for overnight. Next day the samples were again centrifuged and the supernatant was collected and added to the earlier 1 ml extract of each sample. The absorbance of the samples was recorded on microplate reader at 663 nm and 645 nm wavelength. The amount of chlorophyll was calculated using following formula.

\[
\text{Total chlorophyll (mg g}^{-1} \text{FW)} = (20.2 \times \text{OD}_{645} - 8.02 \times \text{OD}_{663}) \times 2 / (1000 \times 0.1)
\]

\[
\text{Chlorophyll ‘a’ (mg g}^{-1} \text{FW)} = (12.7 \times \text{OD}_{645} - 2.69 \times \text{OD}_{663}) \times 2 / (1000 \times 0.1)
\]

\[
\text{Chlorophyll ‘b’ (mg g}^{-1} \text{FW)} = (22.9 \times \text{OD}_{645} - 4.68 \times \text{OD}_{663}) \times 2 / (1000 \times 0.1)
\]

Where, \( \text{OD} \) - optical density
\( \times \) - multiplication

3.2.2.3. Leaf Morphological Analysis

3.2.2.3.1. Clearing leaf tissue

- Terminal leaves of the shoot cultures were harvested and placed inside the glass vials containing 80% (v/v) acetone and thereafter incubated overnight at 4°C. During incubation, the leaf extract was replaced with fresh 80% acetone for the complete removal of chlorophyll pigments from the leaves.
- Cell content of the leaf tissue was removed by dipping leaves in petri-plates (90 mm) containing NaOH (5%; w/v) and SDS (0.1%; w/v) and incubated overnight at 37°C.
- After incubation, leaves were washed several times to remove froth of the detergent adhered over the leaves.
- Samples were taken over glass slides by taking extra precaution to avoid any physical damage to the cleared leaf tissue.
- Without disturbing the specimen, the cleared leaves were mounted in D.P.X. (80-10 g Distrene 5 cm\(^{-3}\) dibutyl phthalate and 35 cm\(^{-3}\) xylene) and examined under compound microscope (Zeiss AXIO fluorescent microscope, Germany) attached with digital camera (Zeiss AxioCAM, Germany).
3.2.3. Estimation of \textit{in Vitro} Accumulation of Ethylene

SNCs were inoculated separately in culture tubes (60 cm$^3$) containing MS basal medium and STS (4 µM) supplemented MS basal medium. These culture tubes were then sealed with silicon rubber caps (Perfit, India) and incubated under \textit{in vitro} culture conditions. \textit{In vitro} production of ethylene by the shoot cultures growing in the culture tubes containing MS basal medium (control) and STS (4 µM) supplemented MS medium were determined at 0, 3, 4, 8, 10 and 12 days after inoculating (dai) nodal explants. On these selected days, 3 ml of gas sample was taken into a hypodermic syringe from the culture tubes (60 cm$^3$) through the silicon rubber cap of the culture tube and analyzed using gas chromatography (model GC 6890, Agilent Technologies, CA, USA), equipped with an FID detector and a Carboxen™ fused silica capillary column 30 m x 0.53 mm (Supelco, PA, USA). Nitrogen was used as the carrier gas at a 5 ml min$^{-1}$ flow rate. The GC oven temperature was programmed to increase from 70 to 200°C at 20°C min$^{-1}$ with a 2 min hold at 70°C and a 5 min hold at 200°C. The ethylene peaks were identified by a retention time of ~ 5.6 min. The standard curve was prepared using different concentrations of pure ethylene (Fig. 3.3) (Sigma Gases, New Delhi, India).

\[ y = 0.849x \]
\[ R^2 = 0.9983 \]

\textbf{Fig. 3.3: Standard curve of ethylene}

3.2.3.1. Relative Growth Study under Varied Ethylene Atmosphere

In order to visualize response exhibited by the control and STS treated plants under ethylene atmosphere, cultures were photographed on 0, 4, 8 and 14 dai. Ethylene induced
culture abnormalities in terms of epinasty, stunted growth, reduced leaf size and, aerial root and root hair development for shoots growing on MS basal medium and 4 µM STS supplemented MS basal medium were determined at 14 dai.

3.2.4. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR Analysis) of \textit{StERS1} Gene

Relative transcript levels of \textit{StERS1} gene were determined in shoot cultures growing in the MS basal medium and 4 µM STS supplemented MS basal medium at 14 dai, when ethylene production by the shoot cultures was maximum and its effects were discernible.

3.2.4.1. Sampling for qRT-PCR Analysis

For qRT-PCR analysis, 50-100 mg leaf tissue each from plants growing on MS basal medium and STS supplemented MS basal medium, were harvested at 14 day after inoculation (dai). The same amount of leaf tissue was also harvested from the \textit{in vitro} potato plants growing in their vegetative phase. All the leaf samples were wrapped in aluminium foil and flash frozen in liquid N$_2$ and stored at -80°C for qRT-PCR analysis.

3.2.4.2. RNA Isolation

Total RNA was isolated from using \textit{iRIS} solution following the method of Ghawana \textit{et al.} (2007). The semi quantitative RT-PCR was performed with 2 µg RNA using SuperScript$^{\text{TM}}$ III Reverse Transcriptase (Invitrogen Bioservices, India Pvt. Ltd.) according to the manufacturer’s user manual guide for oligo dT primed first-strand cDNA synthesis in 20 µl reaction on Thermal cycler (S 1000$^{\text{TM}}$; Bio-Rad, Hercules, CA, USA).

3.2.4.2.1. Inactivation of Contaminating RNases
- All the glasswares and plasticwares were dipped overnight in Diethyl pyrocarbonate-treated (DEPC) treated water (un-autoclaved) at 37°C, followed by autoclaving (1.05 Kg cm$^{-2}$). Glasswares and pestle-mortars were baked at 250°C for 4 h. Gel running apparatus was cleaned with 0.5% SDS solution, washed with autoclaved water (add at a final concentration of 0.1% to autoclaved distilled water and mixed well. Keep undisturbed for overnight) and further treated with 3% H$_2$O$_2$ for 1 h and finally, rinsed thoroughly with DEPC-treated autoclaved water.
- All the solutions were prepared in DEPC-treated water and then autoclaved or prepared in autoclaved DEPC-treated water as per autoclaving requirements.
All the experiments were carried out wearing gloves to avoid RNases contamination.

### 3.2.4.2.2. RNA Isolation using *iRIS* Solution

Total RNA was isolated using *iRIS* solution following the method of Ghawana *et al.* (2007).

- Ground 50-100 mg of tissue to a fine powder in liquid nitrogen using pestle and mortar.
- Added 2 ml of *iRIS* solution I (consisting of phenol saturated with Tris (hydroxymethyl)-aminomethane (Tris) buffer to a pH of 6.7 ± 0.2 or pH of 4.5 ± 0.2, 0.3-1% SDS (Ssodium dodecyl sulphate), 0.2-0.8 M sodium acetate (NaOAc) and 10-20 mM EDTA) to the frozen powder and the mixture was further homogenized while still frozen to make a fine paste.
- The mixture was allowed to thaw with intermittent grinding, then 800 μl of *iRIS* solution II (DEPC treated autoclaved de-ionized water having a conductivity of 17-18.2 mega-ohms) was added to the mixture and homogenized further for a while.
- The contents were transferred to 2 ml micro-centrifuge tubes. The tubes were incubated at room temperature (25°C) for 10 min.
- Added 400 μl of chloroform to each tube, vortexed briefly and incubated at room temperature for 10 min. The contents were centrifuged at 13,000 rpm for 10 min at 4°C.
- The upper aqueous phase was transferred into fresh tubes (avoiding contamination at the interphase) and iso-propanol (0.6 volumes) was added to the aqueous phase, vortexed briefly and allowed to settle at room temperature for 10 min.
- The tubes were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was decanted and the RNA pellet was washed with 70% ethanol (in DEPC water).
- The RNA pellet was air dried (avoid over drying) and dissolved in 20 μl of DEPC autoclaved water.
- The total RNA concentration was determined on UV-VIS spectrophotometer at 260 nm and RNA integrity was electrophoretically verified.

### 3.2.4.2.3. Concentrating the RNA

When needed, RNA was precipitated by adding 4 volumes of chilled ethanol in the presence of 0.1 volume of 3 M sodium acetate (pH 4.8) followed by incubation at -70°C.
for 3 h. RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C, rinsed with 70% chilled ethanol and finally dissolved in RNase free water. RNA was quantified as described in Section 3.2.4.2.6.

**Sodium acetate (3M) (Mw = 82.03):** Dissolved 123.05 g of sodium acetate in 300 ml of DEPC (un-autoclaved) water and adjust pH to 5.2 with the help of glacial acetic acid. Final volume was adjusted to 500 ml with DEPC (un-autoclaved) water and autoclaved the solution.

### 3.2.4.2.4. Denaturing Agarose Gel Electrophoresis of RNA

RNA was analyzed on 1.2% denaturing formaldehyde agarose gel (FA gel), to check the integrity by monitoring distinct 28 and 18S rRNA bands. RNA (5 μl) was mixed with RNA loading dye (10 μl) and denatured by incubating the samples at 65 °C in a water bath for 10 min followed by chilling on ice for 5 min. Denatured samples were loaded onto 1.2% formaldehyde agarose gel and electrophoresed at 60 V in 1X FA gel running buffer. The gel was viewed using UV-transilluminator (UV-transilluminator 2000, Bio-Rad Laboratories, USA) and captured on a gel documentation system (Gel Doc™ XR, Bio-Rad Laboratories, USA).

**5X FA Buffer:** 20.9 g of MOPS was dissolved in 750 ml of DEPC (un-autoclaved) water and added 13.3 ml (50 mM) of Sodium acetate (3 M; pH 5.2). Adjusted pH 7.0 with 2 N NaOH and added 10 ml of DEPC treated EDTA (0.5 M; pH 8.0). Made the final volume 1 litre with RNase free water and sterilized by autoclaving.

**1X FA Buffer (500 ml)**

- 5X FA buffer - 100 ml
- DEPC water - 390 ml
- Formaldehyde 37% (12.3 M) - 10 ml

### 3.2.4.2.5. Preparation of Formaldehyde Agarose Gel (Denaturing Gel)

Weighed 600 mg of agarose and dissolved in 10 ml of 5X FA buffer and 32 ml of DEPC-treated water. The agarose was melted by boiling. The flask was cool to 50°C and then added 8 ml of formaldehyde solution. Poured into the gel tray and allowed to solidify for 40 min under a fume hood.

**2X RNA loading dye (for 10 ml)**

- Formamide (95% v/v) - 9.5 ml
SDS (0.025% w/v) - 2.5 mg
Bromophenol blue (0.025% w/v) - 2.5 mg
Xylene cyanol FF (0.025% w/v) - 2.5 mg
Ethidium Bromide (0.025% w/v) - 2.5 mg
EDTA (0.5 mM) - 10 μl from 0.5 M EDTA stock
DPEC treated water to make final volume

3.2.4.2.6. Quantification of RNA

RNA was adequately diluted with DEPC-treated autoclaved distilled water and Spectrophotometric estimation of RNA was done using ND-1000 spectrophotometer (JH BIO Innovations Pvt, India).

- NanoDrop pedestal was washed and the software was opened.
- The instrument was initialized with purified distilled water.
- The blank was then set with 1 μl of the DEPC-treated autoclaved distilled water in which sample was dissolved.
- Sample (1 μl) was added to the lower pedestal, and reading as ng μl⁻¹ was taken at 260 nm and 280 nm as against ‘blank’.
- Data was collected and the pedestal was cleaned with kim wipes.

- RNA purity is indicated by: A260/A280 ratio. Ratio around 1.7-1.9 indicated pure RNA. Ratios lower than this referred to polysaccharides contamination and higher than this value referred to phenol contamination.
- A ratio >1.8 = the DNA sample is contaminated by protein and phenols

3.2.4.3. cDNA Synthesis

- For 20 μl cDNA synthesis, 1 μg of isolated RNA was added to the nuclease free micro-centrifuge tube and volume was made to 7 μl with autoclaved DEPC water. To this mixture, added 1 μl of 10X DNase buffer followed by 1 μl of DNase (Invitrogen Corporation, USA).
- The mixture was incubated in a thermocycler (PCR-S1000™ Thermal Cycler, Bio-Rad Laboratories, USA) at 37°C for 30 min.
• Reaction was stopped by adding 1 µl of 25 mM EDTA (Invitrogen Corporation, USA) to the above mix (in thermocycler) and incubated in a thermocycler at 65°C for 10 min.

• To this added, 1 µl oligo dT (500 ng) primer (12-18 mer) and 1 µl of 10 mM dNTPs mix (DEPC-treated; Invitrogen Corporation, USA).

• Reaction mixture was incubated at 65°C for 5 min and directly transferred into ice for 2 min prior to the addition of 4 µl of 5X First Strand Synthesis buffer, 2 µl 100 mM DTT and 1 µl of SuperScript III Reverse Transcriptase (Invitrogen Corporation, USA).

• The above mixture was incubated at 42°C for 60 min followed by heat denaturation at 70°C for 15 min and finally, stored at -80°C till further use.

• The final concentration and quality of synthesized cDNA was determined spectrophotometrically as described in Section 3.4.1.2.

3.2.4.3.1. Checking cDNA with 26S rRNA Primer Pairs

cDNA synthesis was checked by PCR amplification of 26S rRNA using 26S rRNA based primers developed to be used as internal control in reverse transcription- polymerase chain reaction (RT-PCR; Singh et al. 2004). The details of 26S rRNA primer pair and PCR conditions were mentioned in Table 3.1 and Table 3.3 An aliquot of amplified products were separated on 1.2% (w/v) agarose gel and rest stored at -20°C for further use.

3.2.4.3.2. Electrophoresis of DNA

Agarose gel (1.2%, w/v) was prepared in dH₂O and heated to melt. Ethidium bromide 0.4 µg ml⁻¹ (stock-1 mg ml⁻¹) was added after the solution has cooled down to about 50°C. The gel was casted with required number of comb and left for solidifying. The comb was removed, samples were added and gel was run in 1XTAE buffer at 7 volt cm⁻¹ of gel size.

50X TAE (1 litre)

• 242 g of Trizma base was dissolved in 400 ml of dH₂O.

• 57.1 ml of glacial acetic acid was added to it.

• Added 100 ml of 0.5 M Na₂EDTA, final volume was raised to 1 litre and solution was autoclaved prior to use.
3.2.4.4. qRT-PCR analysis

qRT-PCR analysis was performed on Real-Time PCR system (MX 3000P, Stratagene). The reactions contained 5 µl of 2x Brilliant SYBR Green QPCR master mix (Stratagene), 2.5 µl cDNA (diluted 1:10), 0.5 µl gene-specific forward and reverse primers (5 pm), in a final volume of 10 µl. Primer details were mentioned in Table 3.1 and PCR reaction was carried out as per thermal profile mentioned for StERS1 gene in Table 3.4 A melting curve was performed for each reaction to determine the specificity of the reaction. Reactions were set up in triplicate, including a control with no template (NTC). Ct values were determined as the numbers of amplification cycles needed to reach a fixed threshold in the exponential growth region of the amplification curve. To normalize the variance in cDNA input, S. tuberosum ubiquitin gene (StUb) was used as the internal control in each case (Ducreux et al. 2005). The relative expression ratio of StERS1 in shoot cultures growing in the presence of STS and in vivo plants was calculated with respect to transcript levels of StERS1 in shoot cultures growing in MS basal medium, using REST 2009 software (Pfaffl et al. 2002). Mean values and standard error were obtained from three technical and two biological replicates.

3.3. Regeneration of Adventitious Shoot Buds from Internodal and leaf Explants

Internodes and leaves were sourced from aseptic and healthy shoot cultures growing on STS (4 µM) supplemented MS basal medium were used as an explant for in vitro shoot regeneration studies. For each experiment, leaves and internodes were excised with sterile blades in LAF cabinet and transferred on 90 mm Petri-plates (High Media, India) containing 25 ml MS media supplemented with varied concentrations and types of PGRs. Petri-plates inoculated with internodal explants were sealed with parafilm and incubated under standard culture conditions mentioned earlier. For the sake of comparison the work done by Mr Awadhesh Kumar Pal on another potato cultivar being worked in our laboratory i.e. Kufri Sutlej (KS) was also considered and discussed side by side in terms of shoot bud regeneration. Regeneration response in both the cvs in terms of callus formation, rhizogenesis, shoot primordia formation and adventitious shoot bud regeneration were observed after every week interval and final regeneration response was recorded 3-4 wks after incubation.
3.3.1. Regeneration Response on Coconut Milk (CM) and Different Plant Growth Regulators (PGRs)

In order to analyze the response of internodal explants to various PGRs, different auxins [2, 4-Dichlorophenoxyacetic acid (2, 4-D) (2.0 mg l⁻¹); indole-3-acetic acid (IAA) (0.1-1.0 mg l⁻¹) and naphthalene acetic acid (NAA) (0.05-0.5 mg l⁻¹)] either singly or in combination with different cytokinins [benzyl amino purine (BAP) (0.1-0.5 mg l⁻¹); kinetin (0.25 mg l⁻¹) and zeatin (3.0 mg l⁻¹)] were tested to achieve shoot bud regeneration. The effect of coconut milk (CM) was studied in the presence of low NAA concentration (0.05 mg l⁻¹). For every PGR combination/s there was a common control in which the internodal explants were placed over MS basal medium. Five replicates of each PGR combination were taken for the study.

CM is an undefined complex mixture of organic substances that has been successfully employed for culturing different plant species (Arditti and Ernst 1993, Suttle 1996, Janarthanam et al. 2011). Coconut milk (CM) contains thermolabile natural organic compounds including PGRs. Therefore, the raw CM after harvesting was filtered through a course filter paper and subsequently through filter assembly fitted with sterile 0.22 μm filter membranes. Aliquots of 20 ml filter sterilized CM were prepared and stored at 4 °C for further use in regeneration studies. The intermodal segments, about 5 mm to 10 mm in length, excised from in vitro shoot cultures and placed on MS basal medium supplemented with 5% (v/v) of raw CM (CM of 5% was prepared by diluting filter sterilized raw CM with sterile dH₂O). PGRs including NAA, BAP, 2,4-D and kinetin were added to the MS basal medium before autoclaving where as, zeatin and IAA were filter sterilized in the LAF cabinet and thereafter, incorporated into the autoclaved media at 40-50°C as per requirement.

3.3.2. Extraction and Estimation of Endogenous IAA

The intermodal segments of both KG and KS showed variable callusing response even when kept in the MS media evincing the presence of endogenous auxin. Therefore, endogenous IAA content in the stem internodes for both the cvs. was estimated and analyzed with respect to shoot bud regeneration.

The intermodal segments were crushed in liquid nitrogen and homogenized in 80% (v/v) chilled methanol (15 ml g⁻¹ FW) containing 100 mg l⁻¹ butylated hydroxytoluene (BHT) and kept overnight at 4 °C. The supernatant obtained after centrifugation at 10,000
revolution per minute (rpm) (Sigma, Model-3K30; Sigma, Labozentruzen, Germany) for 25 min at 6°C was concentrated in vacuo at 30°C, re-suspended in 0.1 M potassium phosphate buffer (pH 8.0) and applied to a polyvinyl pyrolidone (PVP) column. The column (20 cm × 1.5 cm) was eluted with 0.1 M potassium phosphate buffer (pH 8.0). The pH of elute was adjusted to 8.0 and neutral compounds were extracted five times with peroxide-free diethyl ether. The aqueous fraction was acidified to pH 3.0 with 1 N hydrochloric acid (HCl) and partitioned three times against peroxide-free diethyl ether. The ether phases (acidic indole fraction containing IAA) were combined, dried over anhydrous sodium sulfate (Na₂SO₄) and evaporated to dryness in vacuo (30°C). The concentrated extract was re-dissolved in 2 ml of High Performance Liquid Chromatography (HPLC) grade methanol and filtered through 0.45 μm syringe filters (Millipore, Bangalore, India) prior to use.

The analysis was performed on a Shimadzu HPLC (Model LC-20AT pump, DGU-20A5 degasser, CT-10 AS column oven and SIL-20Ac autosampler) equipped with photodiode array detector (CBM-20A; Shimadzu, Kyoto, Japan) and a Phenomenex Luna C18 column (250 × 4.6 mm i.d., 5 μm). The temperature of the column was set at 25 °C. Elution of standard (IAA, Sigma St. Louis, USA) and samples (20 μl) was performed with gradient elution program at a flow rate of 1.0 ml min⁻¹ for 25 min. The mobile phase consisted of 1.54 g l⁻¹ ammonium acetate in H₂O (A) and methanol (B). The HPLC program was: 80% A to 20% A over the first 15 min, from 20% A to absolute methanol (0% A) between 15 to 18 min and finally back to 80% A between 18 to 25 min. The detection wavelength was set at 280 nm. Identification of IAA was performed based on the retention time and spectral matching with the reference standard. For the preparation of calibration curve, standard IAA stock solutions (1.0 g l⁻¹) was prepared in methanol and appropriately diluted to obtain the desired concentrations in the quantification range.
Preparation of 0.1M phosphate buffer

<table>
<thead>
<tr>
<th>pH</th>
<th>Volume of 1 M K$_2$HPO$_4$ (ml)</th>
<th>Volume of 1 M KH$_2$PO$_4$ (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>8.5</td>
<td>91.5</td>
</tr>
<tr>
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<td>9.2</td>
</tr>
<tr>
<td>8.0</td>
<td>94.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Dilute the combined 1M stock solution to 1 litre with distilled H$_2$O.

3.3.3. Effect of TIBA and Zeatin Combinations on Adventitious Shoot Regeneration from Internodal Explants

The IAA content in the internodes of cv. KS was seven times higher than that of cv. KG. To overcome the response of profuse callusing and presence of endogenous IAA, 2, 3, 5-Triiodobenzoic acid (TIBA), an anti-auxin, was tested along with zeatin to achieve adventitious shoot bud regeneration in cv. KG and KS. Twenty four combinations were tested using four concentrations of TIBA (0, 0.5, 1.25 and 2.5 mg l$^{-1}$) and five concentrations of zeatin (0, 0.1, 0.25, 0.5 and 3.0 mg l$^{-1}$). After adjusting the pH to 5.7 all the media mentioned above were autoclaved at 121$^\circ$C and 1.06 kg cm$^{-2}$ for 20 min. TIBA and zeatin being thermolabile were filter sterilized and added to the cooled (~ 45-50 $^\circ$C) MS basal medium media in LAF cabinet. The apical 3-4 internodes of in vitro shoot cultures used as explants and were cultured on 90 mm Petri-plates containing 25 ml regeneration medium. Experiment was conducted with five replicates of each combination and each replicate contains 19 internodal explants (10-15 mm long) per petriplate. Petri-plates inoculated with internodal explants were sealed with parafilm and incubated under standard culture conditions mentioned earlier. Percentage regeneration response and number of shoots regenerated per explant were determined after four wks of incubation.
3.3.4. A Two Step Approach to Achieve Adventitious Shoot Bud Regeneration from Internodal Explants in KG

To achieve optimum shoot regeneration for cv. KG; firstly, internodal explants were placed for 2 wks over step I regeneration medium containing TIBA (0.5 mg l\(^{-1}\)) and zeatin (0.1 mg l\(^{-1}\)) and hitherto referred as PRM1 for initiation of shoot primordia. For the further development of shoot primordia, internodal explants bearing shoot primordia were shifted to nineteen media combinations containing low concentration of NAA (0.0, 0.01, 0.02 and 0.05 mg l\(^{-1}\)) along with zeatin (0.0, 0.125, 1.0, 3.0 mg l\(^{-1}\)). For each PGR combination, there were three replica Petri-plates and each replica petriplate contains 19 internodal explants bearing shoot primordia. Petri-plates inoculated with explants were sealed with parafilm and incubated under standard culture conditions. Percent regeneration response and number of shoots regenerated per explant were determined after 2 wks of incubation in NAA:zeatin media combinations.

3.3.5. Adventitious Shoot Bud Regeneration from Leaf Explants in KG

To achieve regeneration from leaf explants, leaves were taken from 2\(^{nd}\) - 5\(^{th}\) position from the apex of the shoot culture. Basal and terminal portion of the leaves were excised and placed inside Petri-plates containing 25 ml of already derived step-I regeneration medium, PRM1, as discussed in the earlier section (MS basal medium supplemented with TIBA 0.5 mg l\(^{-1}\) and zeatin 0.1 mg l\(^{-1}\)). The Petri-plates were wrapped with parafilm and incubated under standard culture conditions. After 3 wks of incubation, leaf explants were shifted to step-II medium, PRM2, containing NAA (0.01 mg l\(^{-1}\)) and zeatin (3.0 mg l\(^{-1}\)) and incubated for next three wks following standard culture conditions. Percent of leaf explants responding to shoot bud regeneration and number of shoot regenerated per explant were recorded after three wks of incubating leaf explants in PRM2 medium. Experiment was conducted with five replica Petri-plates of PRM1 and PRM2 medium and each replicate contains nine leaf explants per petriplate, for step I and step II, respectively.

3.3.6. Hardening and Establishment of Plantlets in Polyhouses

Regenerated shoots/SNCs were excised and multiplied on a propagation medium comprising of MS medium with 2% sucrose and supplemented with STS (4 µM) and hitherto referred as PM. At two wks stage, profuse rooting was achieved on the PM medium. Plantlets of height 30-40 mm were de-rooted from the PM medium and
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thereafter, roots were cleaned gently giving several washings in tap water. Plantlets were transplanted to plastic pots (diameter 6 cm; height 8 cm) containing sand, soil and farmyard manure (1:1:1) and placed inside hardening chamber (RH, 80%; temperature, 25 ± 2°C) for two wks. Established plants were shifted to contained polyhouses for tuber production.

3.4. Standardization of Transformation Procedures and Generation of Transgenic Plants

3.4.1. Confirmation of Genes in the Construct/Agrobacterium Strain

Once an efficient regeneration system was achieved, the procedure for Agrobacterium tumefaciens mediated genetic transformation using internodal explants was optimized in the cv. KG. E. coli strains harboring separately, CsTLP:pCAMBIA1302 and CsPPO:pCAMBIA1302 constructs (shown in Fig. 3.2) were kindly provided by Dr. Sanjay Kumar, Y. Sreenivasulu, Tejpal Gill, Mr. Asosii Paul. For the present thesis, these constructs were mobilized separately into Agrobacterium tumefaciens GV3101 strain by tri-parental mating. Presence of gene of interest in transformed Agrobacterium tumefaciens strain was confirmed by colony PCR using gene specific primers and PCR conditions mentioned in Table 3.1 and Table 3.3.

3.4.1.1. Plasmid Isolation

Plasmid DNA was isolated from overnight grown E. coli cultures harbouring CsTLP:pCAMBIA1302 and CsPPO:pCAMBIA1302 constructs using the maxi extraction kit (Qiagen, Germany) and described below:

- Single colony was inoculated in 20 ml of LB containing the appropriate selective agent and grown overnight for 16-18 h.
- The culture was grown to mid log phase (16 h) and inoculated about 10 ml of culture into 500 ml of LB medium containing the recommended dose of selection antibiotic. The cells were incubated overnight at 37°C in dark with vigorous shaking at 200 rpm in an incubator shaker.
- The overnight grown cells were harvested by centrifugation at 6,000 rpm for 30 min at 20-25°C and the pellet was suspended in 10 ml of Re-suspension buffer ‘P1’ (containing 50 mM Tris-HCl pH 8), 10 mM EDTA pH 8 and 100 μg/ml RNase A; stored at 4°C) and vortexed vigorously.
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- To the above oakridge tube, 10 ml of the Lysis buffer ‘P2’ (containing 200 mM NaOH and (1% w/v) SDS; stored at room temperature) was added and mixed gently and incubated at room temperature for 5 min.

- Further 10 ml of chilled Neutralization buffer ‘P3’ (containing 3 M potassium acetate, pH 5.5; stored at either room temperature or 4ºC), was added and mixed immediately but gently and incubated on ice for 15-20 min.

- Centrifuged at 13,000 rpm for 30 min at 4ºC and the supernatant transferred promptly with cut end tips into a fresh oakridge (Repeat the above step if the supernatant is not clear).

- Qiagen-tip 500 was equilibrated by applying 10 ml of Equilibration buffer ‘QBT’ (containing 750 mM NaCl, 50 mM MOPS pH 7.0, 15% v/v iso-propanol and 0.15% v/v Triton X-100; stored at room temperature) and the column was allowed to empty by gravity flow.

- The supernatant obtained step was applied to the column and allowed to enter the resin by gravity flow.

- The column was washed twice with 30 ml of Wash buffer ‘QC’ (containing 1 M NaCl, 50 mM MOPS pH 7, 15% v/v iso-propanol; stored at room temperature). The column was allowed to empty by gravity flow.

- Plasmid DNA was eluted from the column by adding 15 ml of Elution buffer ‘QF’ (containing 1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% v/v iso-propanol; stored at room temperature) and collected in a fresh oakridge.

- Plasmid DNA was precipitated with 0.7 volumes of room temperature iso-propanol, and centrifuged immediately at 15,000 rpm for 30 min at 4ºC. Supernatant was removed carefully and the pellet was washed with 5 ml of 70% ethanol, air dried for 5 min and re-dissolved in a suitable volume of TE buffer (10mM Tris-HCl pH 8; 1mM EDTA pH 8.0) and quantified.

- The quality of isolated plasmid was analyzed on 1% (w/v) agarose gel and quantified spectrophotometrically.

3.4.1.2. Quantification of Plasmid DNA

Spectrophotometric estimation of DNA was done using ND-1000 spectrophotometer (JH BIO Innovations Pvt, India).

- NanoDrop pedestal was washed and the software was opened.
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- The instrument was initialized with purified distilled water.
- The blank was then set with 1 μl of the TE buffer in which sample was dissolved.
- Sample (1 μl) was added to the lower pedestal, and reading as ng μl⁻¹ was taken at 260 nm and 280 nm as against 'blank'.
- Data was collected and the pedestal was cleaned with kim wipes.
  
  ➢ A ratio of 1.8 (A₂₆₀/A₂₈₀) = pure DNA sample of best quality
  ➢ A ratio >1.8 = the DNA sample is contaminated by protein and phenols

3.4.1.3. Tri-parental Mating

- Tri-parental mating was performed by doing slight modifications to the protocol described by Hoekema et al. (1983).
- Agrobacterium tumefaciens GV3101 (recipient), E. coli DH5α harboring pRK2013 and E. coli DH5α harboring the plasmid to be mobilized were streaked and grown overnight in 90 mm Petri-plates containing 25 ml of respective media with respective antibiotics to obtain single colonies under the conditions mentioned below:

<table>
<thead>
<tr>
<th>Bacterial culture</th>
<th>Growth medium (soild/liquid)</th>
<th>Incubation temperature</th>
<th>Shaking (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrobacterium (GV3101 without gene of interest; recipient)</td>
<td>YEM + rifampicin (25 mg l⁻¹)</td>
<td>28°C</td>
<td>150</td>
</tr>
<tr>
<td>E. coli DH5α harboring pRK2013 (donor strain)</td>
<td>LB + kanamycin (50 mg l⁻¹)</td>
<td>37°C</td>
<td>150</td>
</tr>
<tr>
<td>E. coli (DH5α harboring the plasmid containing gene of interest to be mobilized)</td>
<td>LB + kanamycin (50 mg l⁻¹)</td>
<td>37°C</td>
<td>150</td>
</tr>
</tbody>
</table>

- One colony each from E. coli DH5α bearing pRK2013, E. coli DH5α harboring the plasmid which has to be mobilized were re-suspended separately in 1 ml of on liquid LB medium. Also, one colony of A. tumefaciens GV3101 strain was re-suspended in 1 ml of liquid YEM medium.
- The bacterial suspensions were vortexed and centrifuged, separately, at 6,000 rpm at RT for 5 min.
- Supernatant was discarded and pellet of all the three bacterial cultures were re-suspended separately in 100 μl autoclaved dH₂O.
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- *E. coli* DH5α bearing pRK2013 helper strain (25 µL), *E. coli* DH5α harboring the plasmid (50 µL) and *A. tumefaciens* GV3101 strain (100 µL) were mixed together in the ratio of 1:2:4.
- The above mix (175 µL) was dropped in the centre of yeast extract (YE) agar petriplate (lacking mannitol) and incubated at 28 °C for 24 h.
- After mating, the bacteria on the YE agar petriplate were scrapped and diluted to tenth volume with autoclaved dH₂O and spread on YEM agar plate with rifampicin (25 mg l⁻¹) and kanamycin (50 mg l⁻¹). The plates were incubated for 48 h at 28 °C.
- Colony PCR of the colonies appearing on the selection plates was performed to confirm the mobilization of gene of interest in the *Agrobacterium* strain GV3101.

3.4.1.4. Colony PCR of Transformed Agrobacterium Strain

Presence of *CsTLP* and *CsPPO* in construct transformed into *Agrobacterium tumefaciens* GV3101 strain was confirmed by colony PCR amplification as below:

- Lysis buffer was prepared with the below mentioned components and autoclaved:
  - Tris-Cl -10 mM
  - EDTA -1 mM
  - Tween 20 -1 %
- A small portion of transformed bacterial colony was picked with a clean microtip and transferred into 50 µL of colony lysis buffer.
- The tubes were incubated in a boiling water bath for 10 min and chilled on ice for 2 min.
- After cooling, cell debris was pelleted by centrifugation for 2 min. Supernatant (colony lysate) was transferred to a new micro-centrifuge tube.
- PCR reaction was set-up as follows

<table>
<thead>
<tr>
<th>Component</th>
<th>Per rxn (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>14.6</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Forward Primer (10 µM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Reverse Primer (10 µM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Colony lysate</td>
<td>2.0</td>
</tr>
<tr>
<td><em>Tag</em> DNA polymerase (5 U/µL)</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20 µL</strong></td>
</tr>
</tbody>
</table>
• Primers and PCR conditions were used as mentioned in Table 3.1 and 3.3.
• The amplified products were analyzed on a 1.2% (w/v) agarose gel.

3.4.2. Standardization of Transformation Protocol

The transformation protocol for cv. KG was standardized for different parameters as described below:

3.4.2.1. Methods of Agrobacterium Infection to Explants

While standardizing the transformation procedure different methods were used to allow the infection of internodal explants with Agrobacterium cells were as follows:

1. Pricking the explants with sterile needle while dipped in the Agrobacterium suspension.
2. Vacuum infiltration for 5 min while by dipping explants in the Agrobacterium suspension.
3. Routinely used method in which explants were dipped for 30 min in Agrobacterium suspension (De Block 1988).
4. Only cut ends of the internodal explants were exposed to Agrobacterium infection.

3.4.2.2. Plant Selection Antibiotic Standardization

For standardization of plant selection dose, filter sterilized hygromycin (plant selection marker antibiotic) was added to the autoclaved regeneration medium (PRM1 and PRM2; mentioned in section 3.3.4.) when it has cooled down to about 40-50°C. The selection doses of hygromycin (0, 5, 10, 15 and 20 mg l⁻¹) were tested by incorporating hygromycin into the regeneration media (PRM1 and PRM2). For each hygromycin concentration there were three replicates and each Petri-plate contains 15-19 explants. Photographs were taken after 4 wks.

3.4.3. Production of CsTLP Transgenic Plants and Molecular Characterization

3.4.3.1. Antibiotic Selection

The internodal segments (10-15 mm) were dipped in Agrobacterium suspension (absorbance, A₆₀₀: 0.6-0.8) for 10 min, blotted dry on sterile Whatman filter paper, transferred onto regeneration medium (PRM1) for 48 h and cultured under dark following standard culture conditions. The co-cultivation continued for two days after which the
transformed explants were washed in sterile distilled water and kept initially for two wks on plant selection step-I regeneration media (PRM1 supplemented with 20 mg l⁻¹ hygromycin and 500 mg l⁻¹ cefotaxime) and after two wks shifted to plant selection step-II regeneration media (PRM2 supplemented with 20 mg l⁻¹ hygromycin and 500 mg l⁻¹ cefotaxime). Putative transgenic shoots obtained on plant selection step-II regeneration media were further screened on MS basal medium containing 20 mg l⁻¹ hygromycin to study the rooting ability of the putative transgenic plants in the presence of hygromycin.

3.4.3.2. Polymerase Chain Reaction (PCR) Analysis of Transgenic Plants

3.4.3.2.1. Genomic DNA Isolation
Genomic DNA from the wild type and putative transgenic lines was isolated as per the modified protocol of Doyle and Doyle (1990).

- Sample (0.5 g) was ground to fine powder after freezing the tissue in liquid nitrogen with the help of a pestle and mortar.
- The powder was transferred to a centrifuge tube containing 4 ml of pre-warmed (65°C) DNA extraction buffer and mixed vigorously by vortexing.
- Incubated the above at 65°C for 1 h with occasional mixing.
- Added equal volume of chloroform: iso-amyl alcohol (24:1) and mixed gently by inverting the tube for 5 min.
- Centrifuged the mixture at 15,000 rpm at 25°C for 20 min.
- The upper layer (aqueous phase) was transferred to fresh centrifuge tube with a wide bore pipette and added 0.6 volume of iso-propanol and mixed by gentle inversions.
- DNA was precipitated by centrifugation at 15,000 rpm at 4°C for 30 min and the pellet obtained was washed with 5 ml of chilled 70% ethanol.
- After draining off the ethanol the pellet was air-dried and finally dissolved in a minimum volume of TE buffer.
- Transferred the above to 1.5 ml eppendorf and added 10 µg ml⁻¹ of the RNase A stock to this, mixed well and incubated at 37°C for 1 h.
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- Added equal volumes of phenol: chloroform: iso-amyl alcohol (25:24:1) and mixed gently by inverting the tube followed by centrifugation at 13,000 rpm for 20 min at 25°C.
- Upper aqueous phase was collected and subjected to chloroform: iso-amyl alcohol extraction.
- Transferred the upper aqueous phase to a fresh tube and precipitated the DNA by centrifugation at 15,000 rpm for 30 min at 4°C after the addition of 0.1 volume of 3M sodium acetate and double the volume of chilled ethanol.

**3M Sodium acetate**
Dissolve 24.609 g sodium acetate in 100 ml of autoclaved distilled water and adjust the pH to 4.8 with glacial acetic acid.

- The pellet was washed once with 70% ethanol to remove the extra salts and after air drying, finally dissolved the DNA in a minimum volume of TE buffer.
- DNA was checked by running on 0.8 % agarose gel in 1X TAE buffer or spectrophotometrically at 260 nm.
- Quantified DNA was stored at -20°C for PCR confirmation.

#### 3.4.3.2.2. PCR Analysis of Putative Transgenic Plants

Introduction of CsTLP in potato cv. KG was confirmed based on PCR amplification with set of primers and PCR conditions mentioned in Table 3.1 and Table 3.3.

#### 3.4.3.3. Southern Hybridization Analysis

**3.4.3.3.1. DNA Isolation**

High quality DNA was isolated from different samples following the method described in Section 3.4.3.2.1.
3.4.3.3.3.2. Restriction Digestion and Electrophoresis

- At least 30 μg of DNA per sample was digested with restriction enzyme (RE; HindIII; New England Biolabs Inc., USA) in a reaction volume of 100 μl compromising the following components:
  
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer (specific to the RE being used)</td>
<td>10 μl</td>
</tr>
<tr>
<td>DNA (30 μg)</td>
<td>50 μl</td>
</tr>
<tr>
<td>Enzyme (15 U μl⁻¹)</td>
<td>6 μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>34 μl</td>
</tr>
</tbody>
</table>

- The reaction mix in the eppendorf tube were tapped briefly and incubated overnight in a circulated water bath at 37°C.
- 3 μl enzyme and 1 μl of 10X buffer were again added in each tube and incubated at 37°C for 5 h.
- 5 μl of the digested samples were run in a thin agarose gel (0.8%, w/v) and checked in UV transilluminator at high intensity for any undigested DNA. If undigested DNA samples were observed, then the samples were incubated for 2-3 h at 37°C.
- The samples if digested properly were vacuum dried in a speedVac (Savant DNA 120 SpeedVac concentrator, Thermo Scientific, USA) till the-volume remains to ≈ 20 μl.
- Agarose gel (0.8 %) was prepared in TAE by boiling agarose in microwave. EtBr (0.5 μg ml⁻¹) was added after cooling agarose to about 45°C and poured in a casting tray with suitable sized comb.
- DNA mixed with loading dye (final volume 1X) was loaded in a proper loading pattern. HindIII digested λ DNA can be used as marker.
- The gel was run on low voltage with a speed of about 2 cm h⁻¹ for 16-24 h.

3.4.3.3.3. Transfer of the Digested DNA to a Nylon Membrane

- The gel was captured in a Gel documentation system (Gel Doc™ XR, Bio-Rad Corporation, USA) but do not overexpose to UV light.
- Transferred the gel to a GEPROTRED and de-purinate by soaking it for 10 min in 100 ml of 0.25 N HCl with gentle shaking on a rocker (25 rpm). After exact 10 min the solution was removed and then rinsed with autoclaved milliQ H₂O to remove HCl completely.
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0.25 N HCl (250 ml):
12.1 N HCl - 5.15 ml
MilliQ H₂O to make the volume 250 ml

- Added 150 ml of denaturing solution and agitated the gel gently on a rocker (25 rpm) for 30 min at room temperature.

Denaturing solution (150 ml):
0.4 N NaOH - 2.4 g
0.6 M NaCl - 5.25 g
MilliQ H₂O to make the volume 150 ml

- After removing the denaturing solution, the membrane was rinse with milliQ H₂O and added 150 ml of neutralizing solution and the gel was kept in it for 30 min at room temperature on a gel rocker (25 rpm).

Neutralizing solution (150 ml):
1.5 M NaCl - 13.14 g
0.5 M Tris (pH 7.4) - 75 ml from 1 M stock
MilliQ H₂O to make the volume 150 ml

- The positively charged nylon membrane was cut (Amersham Hybond™-XL, GE Healthcare, Sweden) slightly larger (1-2 mm) than the size of the gel and soaked in 50 ml of 2X SSC buffer for 5-10 min (avoid touching the membrane with hands).

- Gel tray was placed upside down in another tray containing 20X SSC and place 3 mm blotting sheet wick above the gel tray.

- The wick was saturated by putting 20X SSC with the help of pipette from the tray. Keep on saturating the wick for some time and avoid any air bubbles.

- Slowly placed 5 mm sheets (3-4 sheets) one by one above the wick and saturated with 20X SSC.

- Now transferred the processed gel from the GEPROTRED tray to this and then placed nylon membrane directly above the gel and saturated with 20X SSC from the tray.

- Added 5 mm sheets one by one along with saturating it with 20X SSC, then placed stacks of sheets of 5 and 3 mm and filter paper followed by a weight of 500 g.

- The DNA from the gel was transferred to the membrane by capillary blotting. The process was continued overnight (15-18 h).

- The gel was constantly kept wet with 20X SSC buffer during the transfer. The wells were marked with pencil and then the membrane was removed carefully.
• The nylon membrane was then placed within the folds of the Whatmann No. 3 filter paper and cross linked in a UV cross linker (Hoefer UVC 500, Amersham Biosciences Inc., USA) twice for 2 min each.

• Pre-hybridization, hybridization and immunological detection steps were performed as explained below in Sections 3.4.3.3.5 and 3.4.3.3.6.

20X SSC buffer (Sodium chloride and Sodium citrate)
- 3 M NaCl (Sigma, USA) - 175.3 g/l (Mw 58.44)
- 0.3 M Na-citrate.2H2O - 88.23 g/l (Mw 294.1)

Dissolved in 800 ml of milliQ H2O and final volume made to 1 litre with milliQ H2O after adjusting the pH to 7 with few drops of 14 N HCl. Cover with aluminium foil and autoclave it.

• Diluted 20X SSC buffer with autoclaved milliQ H2O as per the requirement (10X, 6X, 0.5X, 0.1X SSC).

3.4.3.3.4. Preparing the Probe

The following steps were elucidated below:

➢ PCR amplification of the desired fragment with gene specific primers.
➢ Elution of the amplified fragment.
➢ Labeling of the fragment to be used as probe either by non-radioactive or biotin labeling or radio-labeling.

3.4.3.3.4.1. PCR Amplification of Gene of Interest

PCR-amplification of the coding/gene sequence was performed using CsTLP gene specific forward and reverse primers and PCR conditions mentioned in section Table 3.1 and 3.3. Amplified gene product was resolved on 1.2% (w/v) agarose gel.

3.4.3.3.4.2. Elution of Amplified DNA Fragment from Gel

The MinElute Gel Extraction Kit from Qiagen was used as follows:

• The DNA fragment from the agarose gel was excised with a clean, sharp scalpel or razor blade. The excess gel was trimmed away to minimize the amount of agarose and the gel slice was weighed in a tared colourless tube.
• Added 3 gel volumes of the **Gel Solubilization Solution** i.e. **buffer QG** to the gel slice (for every 100 mg of agarose gel, add 300 µl of buffer QG. For >2% agarose gels, add 6 volumes of buffer QG. The maximum amount of gel slice per spin column is 400 mg; for gel slices >400 mg use more than one MinElute column).

• The gel mixture was incubated at 50°C for 10 min, or until the gel slice was completely dissolved. Vortexed briefly, for 2-3 min during the incubation to help dissolve the gel.

• After the gel slice was dissolved completely, check that the color of the mixture is yellow (similar to buffer QG with no gel slice) prior to proceeding to the next step. If the color of the mixture is orange or violet, add 10 µl of 3M sodium acetate (pH 5) and mix until the colour turns yellow.

• Added 1 gel volume of 100% iso-propanol to the sample and mixed until homogenous.

• MinElute column was placed into one of the 2 ml collection tubes (provided) in a suitable rack.

• To bind DNA, the sample was applied to the MinElute column, and centrifuged for 1 min. If the volume of the sample is >800 µl, load the sample onto the column in 800 µl portions and centrifuged for 1 min after loading the column each time.

• Flow-through was discarded and MinElute column was placed back in the same collection tube.

• Added 500 µl of buffer QG to the spin column and centrifuged for 1 min.

• Flow-through was discarded and placed the MinElute column back in the same collection tube.

• Added 750 µl of **Wash Solution** i.e. **buffer PE** to the MinElute column and centrifuged for 1 min.

• Discarded the flow-through and centrifuged the MinElute column for an additional 1 min at 13,000 rpm without any additional wash solution to remove excess ethanol (residual ethanol from buffer PE will not be completely removed unless the flow-through is discarded before the final centrifugation).

• The MinElute column was transferred to a fresh clean 1.5 ml microcentrifuge tube and added 10 µl of buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7-8.5) to the centre of the membrane, let the column stand for 1 min, and then centrifuged for 1
(elution efficiency is dependent on pH and maximum elution efficiency is achieved between pH 7 and 8.5).

- An aliquot of purified DNA was analyzed on 1.2% (w/v) agarose gel. Eluted and purified fragment was quantified spectrophotometrically as described earlier.

3.4.3.4.3. Labelling the Probe
3.4.3.4.3.1. Probe Labeling with Biotin-11 dUTP (Fermentas)

Eluted DNA fragment was quantified spectrophotometrically as described earlier.

- Firstly, chill the following on ice prior to labelling:
  - DNA template to be labelled (about 500 ng) - 10 µl
  - Decanucleotide in 5X Reaction buffer - 10 µl
  - Water, nuclease free - to make volume to 44 µl

- The above components were mixed in 1.5 ml micro-centrifuge tube, vortexed, spin briefly and incubated in a boiling water bath for 5-10 min followed by snap cooling on ice. Spin down quickly.

- Add:
  - Biotin labelling mix - 5 µl
  - Klenow fragment, exo⁻ (5 U) - 1 µl

  Shake the tube and spin down briefly. The tube was incubated for about 20 h at 37°C in a water bath.

- Reaction was stopped by the addition of 1 µl of 0.5 M EDTA pH 8.0.

- The labeled DNA was stored at −20 °C for further use in hybridization.

3.4.3.5. Hybridization

- Nylon membrane was transferred to the hybridization bottle and added 15 ml of pre-hybridization solution into the bottle.

- Membrane was pre-hybridized at 42°C for 2-3 h at 15-30 rpm in hybridization oven.

- Pre-hybridization solution was then replaced with the hybridization solution containing the labeled probe. After denaturing the labeled probe (at 100°C for 10 min, immediately kept on ice and spin), added immediately to the 10 ml of fresh pre-hybridization solution. Membrane was hybridized at 42°C for 16 h at 2-5 rpm.

- Hybridization solution known as “probe” was removed and can be stored at 20°C for 1-2 uses.
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- After hybridization the membrane was subjected to stringency washes at 42°C for 15 min by washing the blots twice with 30 ml 2X SSC buffer containing 0.1% SDS at 15-30 rpm.
- Again the membrane was washed (twice) at room temperature (25°C) for 30 min each with 0.5X SSC buffer containing 0.1% SDS (30 ml) at 15-30 rpm.
- The above buffer was discarded and washed again with 0.1X SSC buffer containing 0.1% SDS (30 ml) for 30 min at 65°C and 15-30 rpm.
- Third wash buffer was discarded and 20 ml of 2X SSC buffer was added for rinsing.
- The membrane was thus, ready to be used directly for immune-detection or can be stored at 4°C.

**Pre-hybridization Solution Prepared in 50% Formamide**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Na2HPO4, pH 7.2</td>
<td>48 ml (0.4 M)</td>
</tr>
<tr>
<td>15 % SDS</td>
<td>48 ml (6 %)</td>
</tr>
<tr>
<td>12 % BSA</td>
<td>10 ml (1 %)</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>120 µl (1mM)</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>10 ml (4.16 mM)</td>
</tr>
<tr>
<td>Formamide</td>
<td>3.88 ml</td>
</tr>
</tbody>
</table>

3.4.3.3.6. Immunological Detection (Biotin Chromogenic Detection Kit)

- After hybridization and stringency washes, the membrane was briefly blot on Whatman filter paper and then incubated for 5 min at room temperature in 30 ml of **Blocking/Washing buffer** on a gel rocker (Rocker 100, Bangalore Genei) with moderate shaking.
- The membrane was blocked in 30 ml of the **Blocking solution** for 30 min at room temperature with moderate shaking.
- Incubated the membrane with moderate shaking in 30 ml of freshly prepared diluted Streptavidin-AP conjugate for 30 min at room temperature.
- The membrane was washed at room temperature with moderate shaking as follows:
  - Incubated twice with 60 ml of **Blocking/Washing buffer** for 15 min.
  - Incubated with 30 ml of **Detection buffer** for 10 min and discard the solution.
- The membrane was then incubated in 30 ml of freshly prepared **Substrate solution** at room temperature in the dark.
The blue-purple precipitate became visible after 15-30 min of incubation.

After colour development, the reaction was stopped by washing in 50 ml of milliQ water for 5 min. The blot was then analyzed for the copy number of introduced gene.

**Blocking/Washing buffer 1X (210 ml):** Dilute 21 ml of the concentrated 10X Blocking/Washing buffer with 189 ml of milliQ H₂O. Diluted buffer can be stored at 4°C for 1 week.

**Blocking solution (60 ml):** Dissolve 0.6 g of Blocking reagent in 60 ml of 1X Blocking/Washing buffer, and stir the suspension on a magnetic stirrer until the Blocking reagent completely dissolves. Shaking of the suspension at 50-60°C facilitates dissolution of the blocking reagent.

**Streptavidin-AP conjugate (30 ml):** Dilute concentrated Streptavidin-AP conjugate 5000 fold by adding 6 µl in 29.994 ml of Blocking solution just prior to use.

**Detection buffer (60 ml):** Dilute 6 ml of 10X Detection buffer in 54 ml of milliQ water. Diluted buffer can be stored at 4°C for 1 wk.

**Substrate solution (30 ml)** Dilute 600 µl of 50X BCIP/NBT solution with 29.4 ml of 1X Detection buffer. The Substrate solution should be prepared just prior to use.

### 3.4.3.4. Slot Blot

The genomic DNA from WT and PCR positive transgenic plants were subjected to slot blot. For this DNA was directly blotted as quantified slots on a positively charged nylon membrane and was probed with a biotin (Fermentas, Life Sciences, Canada) labelled PCR-amplified product of the CsTLP gene. The whole procedure is described below:
3.4.3.4.1. Isolation of Genomic DNA

Total genomic DNA was isolated as per the procedure mentioned in section 3.4.3.4.1.

3.4.3.4.2. Blotting the Membrane

- Nylon membrane (Amersham Hybond™-XL, GE Healthcare, Sweden) was cut to appropriate size of dot/slot blot manifold. DNA sample of 45 ng was taken and diluted, so as to make a total volume of 6 µl each.
- The membrane was placed in 18.2 milliQ water for 5 min with gentle shaking.
- MilliQ water was then replaced with 6X SSC followed by 10X SSC buffer and kept for 5 min each with gentle shaking.
- Blotting assembly (Hoefer PR 648 Slot blot manifold, Amersham Pharmacia Biotech, USA) was set and the screws were tightened after the nylon membrane was placed on manifold.
- Added 100 µl of 10X SSC buffer in each well.
- Vacuum dried at 40-60 mbar, till the nylon membrane dried (about 5 min).
- DNA was denatured by boiling at 100°C for 10 min, snap cooled on ice and centrifuged briefly.
- Denatured DNA samples were loaded on membrane (6 µl) with the help of micropipette and vacuum dried for 2-3 min.
- Added 100 µl 10X SSC buffer in each well and vacuum dried for 5-10 min.
- Blot the membrane on Whatman filter paper no. 1.
- The membrane was then cross-linked twice for 2 min each with the help of UV cross-linker (Hoefer UVC 500, Amersham Biosciences, Inc. USA).
- Membrane was wrapped in a cling film and kept at 4°C till further use or until proceed for hybridization.

20X SSC buffer (Sodium chloride and Sodium citrate)

- 3 M NaCl (Sigma, USA) - 175.3 g/l (Mw 58.44)
- 0.3 M Na-citrate.2H₂O - 88.23 g/l (Mw 294.1)

Dissolved in 800 ml of milliQ H₂O and final volume made to 1 litre with milliQ H₂O after adjusting the pH to 7 with few drops of 14 N HCl. Cover with Al foil and autoclave it. Diluted 20X SSC buffer with
autoclaved milliQ H₂O as per the requirement (10X, 6X, 0.5X, 0.1X SSC).

3.4.3.4.3. Hybridization

The hybridization was performed as mentioned in Section 3.4.3.3.5.

3.4.3.4.4. Labeling of Probe and Immunological Detection

Labelling of probe and detection was performed as described earlier in Section 3.4.3.3.1 and 3.4.3.3.6.

3.4.3.5. Expression Analysis of Transgenic Plants using Semi-Quantitative RT-PCR

The expression of introduced CsTLP gene was checked by semi-quantitative PCR. It includes following steps:

- RNA isolation
- cDNA synthesis
- PCR analysis of synthesized cDNA

3.4.3.5.1. RNA Isolation

RNA was isolated from all the PCR positive CsTLP transgenic lines following the procedure mentioned in Section 3.5.4.2.

3.4.3.5.2. Quantification and Electrophoresis of RNA

Quantification and electrophoresis of RNA was performed as the procedure mentioned in Section 3.2.4.2.6, respectively.

3.4.3.5.3. cDNA Preparation and Semi-quantitative RT-PCR

cDNA synthesis was proceeded following the steps mentioned in Section 3.2.4.3 and 3.2.4.3.1 Equal amount of synthesized cDNA of WT and transgenic plants were used as a template for PCR using CsTLP specific expression primers and PCR conditions mentioned in Table 3.1 and Table 3.3. The expression of 26S rRNA gene by 26S rRNA based primers and conditions mentioned in section were used as internal control for expression studies. The amplified products were checked on 1.2% (w/v) agarose gel as described earlier.
3.4.3.5.4. Visualization of GFP expression

*In-vitro* transgenic plants were de-rooted and roots were rinsed in water to remove agar adhered to the roots. Root tips (5mm) were excised and placed over the glass slide and examined using Confocal Laser Scanning Microscope (Zeiss LSM510 meta Gmbh, Germany) equipped with a Zeiss Axiovert 100 M inverted microscope. GFP auto fluorescence was collected by excitation/emission wave- lengths at 510 nm.

3.5. Phenotypic Analysis of Transgenic Plants

The plants of WT and *CsTLP* transgenic lines (TL1 and TL2), verified molecularly were multiplied under the *in vitro* conditions for phenotypic analysis and stress evaluation. The selection of TL1 and TL2 among the four PCR confirmed transgenic lines was based on single copy insertion of *CsTLP* gene introduced.

Phenotypic analyses of transgensics relative to WT were performed by studying any differences in the shape of microtubers, growth of plants under *in vivo* conditions and tuber characteristics after harvesting.

3.5.1. Microtuber Induction and Morphological Analysis of Raised Microtubers

- SNCs were collected from shoot cultures of WT and transgenic plants and grown for 2 wks on PM medium.
- After two wks of inoculations, shoot cultures were de-rooted and placed inside another autoclaved flask containing liquid microtuber induction medium (MIM).
- For microtuber induction, shoot cultures in MIM were incubated for three wks inside growth chamber under dark conditions at 20 ± 2°C.
- The microtubers were harvested and analyzed for any morphological changes.

**MIM** - MS basal medium with 8.0% sucrose, supplemented with BAP (2.5 mg l⁻¹) and chlorocholine chloride (CCC; 250 mg l⁻¹).

3.5.2. Phenotypic Analysis of Transgenic Plants Grown in Polyhouse

*In vitro* WT and *CsTLP* transgenic plants (TL1 and TL2) were hardened successfully using the procedure mentioned earlier (**Section 3.3.6.**) and transplanted into plastic pots and placed inside the contained polyhouse to study any differences in the growth of TL1 and TL2 relative to WT. The growth parameters that were recorded after three month of
planting were leaf area, internodal length as well as thickness and root volume. After harvesting the tuber characteristics viz. tuber volume (cm$^3$) and average number of eyes per tuber were recorded. Plants were also analyzed stringently both during their vegetative growth and at the time of harvesting for any disease incidence. Minitubers were sorted on the basis of size and free of any disease incidence and kept in cold store set at 4°C for further stress analysis.

3.5.2.1. Root Volume

Plants were hand lifted after being wrenched at a 25 cm soil depth. Stem portions were excised and roots were washed under tap water without damaging the root system. Pre-fill measuring cylinder with known volume of distilled water and this represented initial volume ($V_1$). Root system was immersed in a measuring cylinder already filled with known volume of distilled water ($V_2$) and rise in the volume of water in the cylinder was recorded as $V_2$. Root volume was calculated by following formula

$$\text{Root volume} = \text{water displacement} = (V_2 - V_1)$$

3.5.3. Identification of Fungal Pathogen Infecting Tubers

At the time of harvesting, it was observed that approximately 30% tubers of WT plants were rotted due to fungal infection while TL1 and TL2 tubers remained un-infected. This interesting observation prompted us to characterize the causal organism of rot.

3.5.3.1. Isolation of Pathogen

- Infected tubers were collected and cleaned to remove any soil particles adhered with tubers.
- Under LAF, infected tubers were swabbed with 70 % (v/v) ethanol followed by rinsing with several changes of sterile distilled water and later on dried over sterile filter paper.
- The potatoes were cut into small blocks of pith region of tuber (1.5 cm) and placed onto potato dextrose agar (PDA) medium. The same procedure was followed for un-infected tuber separately. The plates were incubated at 25 °C for 48 h.
- After incubation, pure culture was maintained on PDA medium for further characterization.
3.5.3.2. Pathogenicity Test of the Isolated Pure Culture

- WT potato tubers were used in this experiment. Initially, tubers appearing healthy and uniform in size (100-120 g) were selected and washed to remove excess soil adhered with tubers.

- Under LAF cabinet, tubers were surface sterilized in 0.5% sodium hypochlorite solution for 10 min and rinsed in three changes of sterile distilled water (Lui and Kushalappa 2002; Lui et al. 2005). After it, tubers were swabbed with 70% (v/v) ethanol followed by rinsing with several changes of sterile distilled water and later on dried over sterile filter paper.

- The surface sterilized tubers were excised transversely resulting slices of 10 mm thickness. These slices were taken over sterile Petri-plates.

- Isolated pure fungal culture was placed in the middle of slice and Petri-plates were wrapped using parafilm. Petri-plates were incubated for 1 wk under dark at temperature 25°C to test the pathogenicity of pure culture.

3.5.3.3. Molecular Characterization of Fungus

3.5.3.3.1. Fungal DNA Isolation

Mycelium from 3-day old colony was scarped from the petriplate, frozen in liquid nitrogen, and ground to fine powder. Genomic DNA was isolated using Qiagen Plant DNeasy Kit (Qiagen Gmb H, Hiden).

3.5.3.3.2. PCR Amplification of Internal Transcribed Spacer (ITS) Region

Pure fungal culture was identified on the basis of ITS region sequencing. For this, fungal DNA was used as a template and ITS rDNA (Fig. 3.4) was amplified using ITS1 and ITS4 primers (White et al. 1990). The details of primer and PCR conditions were mentioned in Table 3.1 and Table 3.3.

![Fig. 3.4: Annealing sites for the primer pair used in the study (drawing not to scale)]
3.5.3.3.3. Gel Extraction

Amplified ITS region (∼600 bp) was electrophoresed on a 1.2% agarose-ethidium bromide (EtBr) gel in 1X TAE buffer and eluted as per the procedure mentioned in Section 3.4.3.3.4.2.

3.5.3.3.4. Cloning

The amplified and gel extracted PCR products were ligated into a pGEM®-T Easy vector (Promega, USA). Vector map and the site of cloning of insert in pGEM®-T Easy vector (Promega, USA) is shown in Fig. 3.5. The pGEM®-T Easy vector is a linearized vector with 3′ terminal thymidine at both ends (Fig. 3.5). The T-overhangs at the insertion site greatly improve the efficiency of ligation of the products by preventing re-circularization of the vector and providing a compatible overhang for PCR products generated by Taq polymerase.

Fig. 3.5: Map of pGEM®-T Easy vector (a), the promoter and multiple cloning sequence of the pGEM®-T Easy vector (b) (adapted from www.promega.com)
*Taq* polymerase often adds a single deoxyadenosine, in a template-independent fashion at 3’-ends of the amplified fragments. The pGEM®-T Easy vector is 3018 bp high copy number vector containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide code allows the recombinant clones to be directly identified by color screening on indicator plates.

Ligation reaction was set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Rapid ligation buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>pGEM®-T Easy vector (50 ng)*</td>
<td>1.0</td>
</tr>
<tr>
<td>PCR product (~ 450 ng)</td>
<td>X</td>
</tr>
<tr>
<td>T4 DNA ligase (3 Weiss units/ µl)*</td>
<td>1.0</td>
</tr>
<tr>
<td>Deionized H₂O</td>
<td>Y</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10.0</strong></td>
</tr>
</tbody>
</table>

*Supplied by the manufacturer*

Ligation reaction was mixed by pipetting and incubated overnight at 4°C for 16 h before proceeding for transformation in competent *E. coli* cells.

### 3.5.3.3.5. Preparation of Competent DH5α *E. coli* Cells

Competent DH5α *E. coli* cells were prepared as follows:

1. DH5α *E. coli* cells were removed from the vial with a sterile pipette tip, and streaked on LB agar plate.
2. The plate was incubated overnight at 37°C.
3. A single colony was picked, inoculated in 10 ml of LB medium and allowed to grow overnight at 37°C.
4. One ml of overnight grown culture was added to 100 ml pre-warmed LB medium in a 250 ml flask and incubated on shaker at 37°C until an optical density at 600 nm (OD₆₀₀) of 0.5 was obtained (it takes approximately 90-120 min).
5. The culture was cooled on ice for 5 min and transferred to a sterile round-bottom centrifuge tube.
6. The cells were collected by centrifugation at low speed (5 min, 6000 rpm; 4°C).
7. Supernatant was discarded carefully.
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8. The cells were re-suspended gently in 30 ml of cold (4°C) TFB1 buffer and kept the suspension on ice for an additional 90 min.

9. The cells were collected by centrifugation (5 min, 6000, 4°C).

10. The supernatant was discarded carefully and the cells were re-suspended carefully in 4 ml ice-cold TFB2 buffer.

11. Aliquots of 100μl were prepared in sterile micro-centrifuge tubes and frozen in liquid nitrogen.

12. Competent cells were used for transformation or stored at -80°C till further use.

**TFB1**: 100 mM RbCl, 50 mM MnCl₂, 30 mM potassium acetate, 10 mM CaCl₂, 15 % glycerol, pH 5.8; sterile-filter.

**TFB2**: 10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂, 15 % glycerol, adjust to pH 6.8 with KOH, sterile filter.

**Competent cell buffer**: Dissolved 100 mM CaCl₂, 70 mM MnCl₂, 40 mM CH₃COONa (pH 5.5) in water and autoclaved.

3.5.3.3.6. Transformation of Competent Bacterial Cells

1. Ligation mix (5 μl) was transferred into a cold sterile 1.5 ml micro-centrifuge tube, and kept on ice.

2. DH5α competent cells were thawed on ice.

3. The cells were resuspended and 100 μl of the cell suspension was transferred into the micro-centrifuge tube with the ligation mix by mixing carefully. It was kept on ice for 20 min.

4. The tube was transferred to a 42°C water bath or heating block for 45 s.

5. Psi Broth (500 μl) was added to the cells and incubated for 4-5 h at 37°C.

6. Twenty μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (20 μg ml⁻¹) was plated onto LB plates containing 100 μg ml⁻¹ ampicillin and allowed to incubate for 30 min in the laminar flow cabinet.

7. Transformation cultures (200 μl) were plated on the above plates and incubated at 37°C overnight.

**Psi broth**: Dissolved 4 mM MgSO₄, and 10 mM KCl in LB medium
3.5.3.3.7. Colony PCR

All the white (transformed) colonies were re-streaked on a fresh ampicillin plate and colony PCR was performed to check the cloning of PCR product as follows:

1. A small portion of transformed bacterial colony was picked up with a sterile microtip and transferred into 50 µl of colony lysis buffer. The tubes were incubated in a boiling water bath for 10 min and chilled on ice for 2 min. After cooling, cell debris was pelleted by centrifugation for 2 min. Supernatant (colony lysate) to be used as a template was transferred to a new micro-centrifuge tube PCR. The presence of ITS fragment of 583 bp was confirmed by colony PCR using vector specific M13 forward (5’-GTA AAA CGA CGG CCA GTG-3’) and M13 reverse (5’- GGA AAC AGC TAT GAC CAT G -3’) primers flanking the cloning site.

2. PCR was set-up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile dH₂O</td>
<td>14.3</td>
</tr>
<tr>
<td>10X PCR buffer*</td>
<td>2.0</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>0.5</td>
</tr>
<tr>
<td>M13 Forward primer (10 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>M13 Reverse primer (10 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Colony lysate</td>
<td>2.0</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/µl)*</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20.0</strong></td>
</tr>
</tbody>
</table>

*Supplied by the manufacturer.

3. The PCR parameters were: 25 cycles of 94°C for 30 s, 54°C for 40 s, 72°C for 2 min and final extension at 72°C for 7 min, and cooling to 4°C.

4. PCR amplified products were electrophoresed on 1.2% (w/v) agarose-EtBr gel for analysis.

3.5.3.3.8. Plasmid DNA Isolation

PCR-positive colonies were grown in 5 ml of LB media with appropriate antibiotics and incubated at 37 °C overnight with shaking at 150-200 rpm. Plasmid DNA was isolated using maxi extraction kit (Qiagen, Germany) and described earlier in Section 3.4.1.1.
3.5.3.9. DNA Sequencing

DNA sequencing was performed using BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, USA) using M13F or M13R primer. Sequencing reaction was setup as described below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA (200-400 ng)</td>
<td>X</td>
</tr>
<tr>
<td>Primer (10 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Big Dye® Terminator 5X sequencing buffer*</td>
<td>1.0</td>
</tr>
<tr>
<td>Big Dye® Terminator sequencing RR100®</td>
<td>1.0</td>
</tr>
<tr>
<td>Deionized H$_2$O</td>
<td>Y</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>5.0</strong></td>
</tr>
</tbody>
</table>

*Supplied by the manufacturer.

The samples were subjected to 25 cycles of 95°C for 10 s, 54°C 30 s and 60°C for 4 min in a GeneAmp® PCR System 9700 (Applied Biosystems, USA).

3.5.3.10. Purification of Sequencing PCR Product

PCR products were purified by Montage SEQ96 Cleanup Kit (Millipore Corp., USA). Injection Solution (20 µl) (provided in kit) was added to each reaction tube and mixed gently by pipetting. All of the mixture was transferred to the bottom of the wells of the SEQ96 plate. The SEQ96 plate was placed on a vacuum manifold (Millipore corp., USA). The vacuum was set to 23-25" Hg. Vacuum was applied until all the wells were completely empty. The vacuum source was shut off and the SEQ96 plate was removed from the manifold. Excess liquid was blotted off from the bottom of the SEQ96 plate by briefly pressing the plate on a paper towel. Injection Solution (25 µl) was added to the bottom of each well of the SEQ96 plate and the plate was placed back on the vacuum manifold and vacuum was applied until the wells were completely empty. The step was repeated again. Injection Solution (25 µl) was added to each well in the plate and the PCR products were resuspended by pipetting up and down 20-25 times. The products were transferred to tubes/96 well optical plates. The plates were then loaded on an ABI PRISM™ 310 Genetic Analyzer or ABI 3130xl Genetic Analyzer (Applied Biosystems, USA) for analysis.
3.5.3.3.11. Bioinformatic Analyses

The sequence was analyzed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov) search algorithm and aligned to the nearest neighbors (Altschul 1997). The phylogenetic tree was constructed using the MEGA4 software package after aligning the sequences with ClustalW (Chenna et al. 2003) and generating evolutionary distance matrix, inferred by the neighbor-joining method using Kimura’s two-parameter model (Kimura 1993).

3.6 Evaluation of Transgenic Plants for Stress Analysis

TLPs are known to provide biotic and abiotic stress tolerance therefore in the present study transgenic potato plants, TL1 and TL2, overexpressing CsTLP were analyzed for fungal stress and water stress tolerance. The selection of TL1 and TL2 among the four PCR confirmed transgenic lines (TL1-TL4) was based on single copy insertion of CsTLP gene.

3.6.1. Tuber Bioassay

Tuber bioassay was developed in our lab to screen transgenic tubers against M. phaseolina and the whole procedure is described below:

- Healthy and uniform tubers of WT and CsTLP transgenic plants (TL1 and TL2) were randomly selected and washed thoroughly under tap water without injuring the tuber peel.
- Under sterile conditions, the tubers were swabbed gently with 70% (v/v) ethanol followed by rinsing in autoclaved distilled water and finally air dried.
- Mycelial mat of M. phaseolina culture grown on PDA medium at exponential phase was sliced into pieces of equal size (6×4×3 mm).
- Tubers of WT, TL1, and TL2 plants were punctured (6 mm long and 5 mm deep) using sharp surgical blade and inoculum was inserted into the periderm. Punctured surface of tubers was sealed by pressing the excised portion transversely followed by coating of molten wax (45°C).
- Tubers inoculated with sterile PDA medium were used as reference for experimental procedure.
- To provide a reference for the healthy tuber skin, tubers of WT, TL1, and TL2 were left intact throughout out the experiment and referred as non-inoculated controls.
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- All the inoculated, mock inoculated and non-inoculated tubers were incubated at 25°C for 3 wks. After 3 wks, inoculated tubers (WT, TL1, and TL2) along with their respective controls were sliced into two sections through the middle and photographed to demonstrate inner surface damage caused by *M. phaseolina*.
- Damage to the sprouts/lateral eyes and discoloration of tubers was also recorded.
- The experiment was repeated three times over two season crops, consisting of five WT and CsTLP transgenic tubers for each replicate.

3.6.2. Effect of Leaf Extract from Transgenic Plants on *M. phaseolina* Mycelial Growth

- Leaf tissues (1 g each) from WT and CsTLP transgenic (TL1 and TL2) plants growing under contained polyhouse were harvested, crushed in 0.1 M potassium phosphate buffer (pH 7.0) and incubated at room temperature for 30 min.
- The leaf extract was centrifuged at 10,000 rpm and the supernatant was collected in fresh falcon tube.
- Supernatant was filter sterilized, aliquoted to 14 ml Falcon polypropylene round-bottom tube (Tarsons, India) and stored at -20°C.
- Individually, 1 ml of filter sterilized leaf extract of WT, TL1 and TL2 was added to 90 ml PDA after cooling the medium to about 45 °C followed by pouring equal volume (20 ml) into five Petri-plates (90 mm).
- As a control, 100 ml PDA medium was equally poured into five Petri-plates. For bioassay controls, same volume (1 ml) of phosphate buffer, boiled crude protein extract of leaves of WT and CsTLP transgenic plants were added to the PDA medium as mentioned above.
- Uniform size mycelial disks of *M. phaseolina* with diameter (5 mm) were inoculated in the centre of all the controls and leaf extract-amended PDA medium.
- All the Petri-plates were incubated at 28°C for 72 h.
- After incubation, growth zone diameter of the fungal colony in cms was measured using measuring scale and photographed.
- The experiment was repeated three times with five replicates for each control and treatment.
3.6.3. Challenging Transgenic Plants Against *Phytophthora infestans*

3.6.3.1. *P. infestans* Strain, Maintenance and Inoculum Preparation

Virulent strain of *P. infestans* was obtained from CPRI, Shimla and maintained by sub-culturing on Rye medium (Caten and Jinks 1968) Petri-plates. The Petri-plates were incubated in dark at 18°C. To maintain the pathogenicity, *P. infestans* after 3rd generation on Rye medium was cultured over tuber slices of highly susceptible potato cv. Kufri Chandramukhi (KCM)

3.6.3.1.1. Rye Agar Medium Preparation for 1 litre

1. Soaked 60 g of rye grain in distilled water for 24 hours at room temperature. This is done in a small tray so that water just covers grain. Cover tray tightly with aluminum foil.
2. Next day, pour supernatant off germinated grain and put aside.
3. Place grain in a beaker, add distilled water (about 1 inch above grain) and blenderize on high for 2 min. Cook in water bath for 1 h at 68°C. Don’t modify extraction time or temperature.
4. Filtered through 4 thicknesses of cheese cloth squeezing gently to remove residual liquid. Discard cheese cloth and grain sediment.
5. Combine original supernatant (liquid poured off grain at the beginning) with filtrate. (At this point the preparation can be frozen for use later).
6. Add 20 g sucrose, 15 g agar then adjust volume to 1 liter.
7. Autoclave at 15 psi for 20 min and Petri-plates stored at 4°C.

3.6.3.1.2. Inoculum Preparation

1. Mycelia were scooped from the two wks old *P. infestans* culture growing over slice of KCM and rinsed in sterile distilled water to release the sporangia.
2. The mycelial/sporangial suspension was stirred with a magnetic stirrer for 10 min.
3. The suspension was strained through four layers of cheesecloth and incubated at 4°C for 4 h to release zoospores.
4. Prior to inoculation the concentration was adjusted to $1 \times 10^5$ zoospores ml$^{-1}$ based on haemocytometer readings.
3.6.3.1.3. Detached Leaf Assay

- Primary leaves were excised from the third to the sixth node of six wks old WT and CsTLP transgenic (TL1 and TL2) plants.
- Each leaf was inoculated with a single droplet (25 µl each) of zoospores on the abaxial side. As a control, leaves of highly susceptible potato cv. KCM were also inoculated with a same amount of inoculum.
- The leaflets from each set of plants were placed in separate trays and incubated in controlled-environment chambers under dark conditions (temperature 18 ± 2°C and RH > 90 %). Throughout the incubation period RH close to 90-95% was maintained.
- The degree of infection was analysed visually by the appearance of *P. infestans* induced necrotic lesion at 2, 5 and 10 days post inoculation (dpi).

3.6.4. Defense-Related Gene Expression in the CsTLP Transgenic Potato Tubers upon *M. phaseolina* Infection

Activation of defense response in terms of transcript accumulation of defense response phenylpropanoid pathway gene (*StPAL*), LOX pathway gene (*StLOX*), and native *S. tuberosum* TLP gene (*StTLP*) in *M. phaseolina* inoculated tubers was monitored by qRT-PCR assay in a time course experiment.

3.6.4.1. *M. phaseolina* Inoculation

- Healthy tubers of potato tubers were collected and cleaned to remove any soil particles adhered with tubers.
- Under LAF, infected tubers were swabbed with 70% (v/v) ethanol followed by rinsing with several changes of sterile distilled water and later on dried over sterile filter paper.
- Surface sterilized plants were sliced into 10 mm thick sections using sterile blade.
- Uniform size (diameter 5 mm) *M. phaseolina* culture disks were placed in the centre of all the tuber slices.
- As a control; tubers were mock inoculated with sterile PDA medium disks of diameter 5 mm.
- Petri-plates were sealed with parafilm and incubated at 25 °C. Samples (50 mg tuber tissue) were harvested before inoculation i.e day zero and around the inoculated
portion of tuber at 2, 4 and 8 day post inoculation (dpi). As a control, samples from mock inoculated tubers were also harvested.

- All the samples were frozen in liquid nitrogen followed by their storage at -80 °C for RNA isolation and further qRT-PCR analysis.

### 3.6.4.2. RNA Isolation and cDNA Synthesis

RNA isolation was performed according to method followed in section 3.2.4.2.2. Quality and quantity of RNA was checked by denaturing agarose gel electrophoresis and UV-VIS spectrophotometer at 260 nm, respectively as mentioned in Section 3.2.4.2.6. cDNA preparation was proceeded as per the mentioned details in Section 3.4.3.5.3. Synthesis of cDNA was checked using 26S rRNA primers and PCR conditions mentioned in Table 3.1 and 3.3.

### 3.6.4.3. qRT-PCR analysis

qRT-PCR analysis was performed on Real-Time PCR system (MX 3000P, Stratagene). The reactions contained 5 µl of 2X Brilliant SYBR Green QPCR master mix (Stratagene), 2.5 µl cDNA (diluted 1:10), 0.5 µl gene-specific forward and reverse primers (5 pm), in a final volume of 10 µl. qRT-PCR primer details were mentioned in Table 3.2 and PCR reaction was carried out as per thermal profile mentioned for defense response pathway genes (StLOX, StPAL, StTLP) and CsTLP in Table 3.4. A melting curve was performed for each reaction to determine the specificity of the reaction. Reactions were set up in triplicate, including a control with no template (NTC). Ct values were determined as the numbers of amplification cycles needed to reach a fixed threshold in the exponential growth region of the amplification curve. To normalize the variance in cDNA input, S. tuberosum ubiquitin gene (StUb) was used as the internal control in each case (Ducreux et al. 2005). The relative expression ratio at 2, 4, and 8 dpi in WT and CsTLP transgenics (TL1 and TL2) was calculated with respect to mock inoculated control using REST 2009 software (Pfaffl 2002). To study the role of CsTLP overexpression on the transcriptional changes in intrinsic potato defense response genes, relative expression ratio was determined in TL1 and TL2 tubers before and after inoculation (8 dpi) with M. phaseolina relative to WT tubers before and after inoculation, respectively. Mean values and standard error were obtained from three technical and two biological replicates.
3.6.4.4. Bioinformatic Analysis of Higher StTLP Expression in Both WT and CsTLP Plants

The nucleotide homology of StTLP primer set (Table 3.2) was identified by NCBI searches on the non-reductant (NR) database. The searching parameters were: maximum identity as well as query coverage >95%. Using the above mentioned parameters we obtained one significant hit corresponding to Solanum tuberosum (AY737315.1) corresponding to putative Thaumatin-like protein gene (PR-5/319). The obtained nucleotide hit was mapped to their physical position in the genome using the pseudomolecules (version 3) provided by the PGSC (Potato Genome Sequence Consortium) (Solgenomics.net/organism/Solanum tuberosum/genome).

3.6.5. Evaluation of Transgenic Plants for Drought Tolerance in Pots

3.6.5.1. Experiment Layout

Uniform size (60-70 cm$^3$) and weight (40.0-50.0 g) tubers of WT plants along with CsTLP transgenic lines TL1 and TL2 plants were selected and sown at a depth of 7 cm, one in each pot (30 cm × 30 cm) containing sandy-loamy soil. Plants emerged within 10-15 days and were maintained with regular irrigation in the month of February-May in the transgenic containment facility of the institute as approved by the competent authority. After five wks of sowing, transient water stress was imposed by withholding water for 3, 6, 9 and 12 days. Last day of the 5th week when the soil was fully saturated with water was considered as a zero day well watered control. The average day/night temperature during the transient water stress experiment (0-15 days of water withholding) was 24/12°C and average RH was 42%. Eighteen uniform plants of almost equal height were selected for each of WT and CsTLP transgenic potato plants. Three plants each for 0, 3, 6, 12 and 15 water stress duration were considered for this study. Physiological data and samples for biochemical analysis were collected on the assigned days of water withholding and well irrigated control.

3.6.5.2. Physiochemical Analysis of Soil Parameters

3.6.5.2.1. Determination of Soil pH

For soil pH determination, following method was used as described below:

1. Take 1:2 soil water suspension for which pH is to be determined.
2. Switch on the pH meter and allow the pH meter to warm up for 15 minutes to eliminate asymmetric potential of the instrument.

3. Put the standard buffer solutions for calibration of pH meter. The pH meter automatically recognize and calibrates to these standard buffer value which makes pH calibration faster and easier.

4. The electrodes are then immersed in the beaker containing soil paste or soil water suspension and read pH on the dial and record it.

5. Remove the electrodes from the soil paste/or soil suspension, clean them with distilled water and then dip into a beaker of distilled water. The electrodes are maintained in working condition by keeping them immersed in distilled water.

**Standard buffer solutions:** These may be of pH 4.01, 7.0 and 10.01 buffer standard. To prepare buffer solution, in case of buffer tablets (available in the market) a single piece is dissolved in freshly prepared double distilled water and volume is made up to 100 ml.

### 3.6.5.2.2. Estimation of Available Nitrogen

- Take 2 gm soil in a dry Kjeldahl flask and add 20 ml of distilled water to mist the soil.
- Add 50 ml of 0.32% KMnO<sub>4</sub> and 50 ml 2.5% NaOH and paraffin wax (≈ 1-2g)
- In the distillation (receiving) end, keep a 250 ml conical flask containing 2 ml 2% boric acid and 5-6 drops of mixed indicator to collect the released ammonia.
- Start distillation by using automatic distillation unit for 9-12 min (nearly 100 ml of distillate to be collected)
- Pinkish colour turns to light green by the absorption
- Titration with 0.02 N H<sub>2</sub>SO<sub>4</sub>.
- End point: Green colour changes to pinkish colour (initial colour).

**Calculation:**

\[
\text{Available N (kg ha}^{-1}) = \frac{V \times 0.02 \times 0.014 \times 2.24 \times 10^6}{W.S}
\]

*Where,*

\[V = \text{Volume of 0.02N H}_2\text{SO}_4 \text{ required for titration}\]

\[W.S = \text{Weight of soil sample taken}\]
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- KMNO₄ solution (0.32%) – Dissolve 3.2 g of KMNO₄ in distilled water, and make up the volume to 1 litre.
- NaOH (2.5%) – Dissolve 100 g of sodium hydroxide pellets in distilled water, and make up volume to 1 litre.
- H₂SO₄ - 0.02 N
- 2% Boric acid solution containing 20 ml of mixed indicator per litre.
- Mixed indicator- 0.066 g methyl red plus 0.099 g bromocresol green dissolved in 100 ml of 95% alcohol.

3.6.5.2.3. Estimation of Available Phosphorus

Available phosphorus was estimated using the method Mehlich No.3 (Mehlich 1984). An extractant known as Mehlich No.3 was introduced as a universal extractant.

1. Weigh 2.5 g of <0.2mm dry soil into a 150 ml Erlenmeyer flask.
2. Add 25 ml of extracting solution (soil to solution ratio 1:10).
3. Stopper and shake the suspension for exactly 5 minutes on reciprocating shaker (180 + oscillations/min). Immediately filter it.
4. Place a 1 ml aliquot of the extract in a 25 ml volumetric flask. Add distilled water to 20 ml and then add 4 ml of reagent B. after 10 minutes, read the intensity of blue colour at 882 nm.

Calculation

Available P (ppm) = \( \frac{R \times 25 \times 25}{2.5 \times 1} \)

- 0.2 N acetic acid, 0.25 N ammonium nitrate, 0.013 N HNO₃ and 0.015 N NH₄F 0.001M EDTA
- Reagent A. Dissolve 12 g ammonium molybdate \((\text{NH₄})₆\text{Mo}_7\text{O}_{24}.4\text{H}_2\text{O})\) in 250 ml of distilled water. Dissolve 0.2908 g antimony potassium tartrate \((\text{K(SbO})\text{C}_4\text{H}_4\text{O}_6.1/2\text{H}_2\text{O})\) in 100 ml water. Add these two solutions to 1000 ml of 2.5 MH₂SO₄, mix thoroughly and make to 200 ml. Store in pyrex glass bottle in a dark and cool place.
- Reagent B. Dissolve 1.056 g ascorbic acid \((\text{C}_6\text{H}_8\text{O}_6)\) in 200 ml reagent A and mix. This does not keep for more than 24 h at room temperature. Prepare daily as required.
- Sulphuric acid 2.5M: Dilute 140 ml of conc. H₂SO₄ to 1 litre.
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- Standard stock P solution: Dissolve exactly 0.439 potassium dihydrogen orthophosphate (KH$_2$PO$_4$) A.R. grade in half a litre of distilled water. Add 25 ml of 7N H$_2$SO$_4$ (approx) and make to one litre with distilled water. This gives 100 ppm P standard stock solution. From this, a 2 ppm solution is made by diluting it 50 times.

**Standard Curve**
To prepare the standard curve, 1, 2, 3, 4, 5 and 8 ml of 2 ppm P solution are taken in 25 ml volumetric flasks. To these, 5 ml of the extracting solution (Mehlich No.3) is added. Added distilled water to make the volume to 20 ml and then added 4 ml of reagent B. Make the volume with distilled water and mix. Prepare a blank with Mehlich No.3 solution, distilled water and 4 ml of reagent B. After waiting for 10 minutes, read the intensity of the blue colour on a spectrophotometer at 882 nm (Watanabe and Olsen 1965).

3.6.5.2.4. Estimation of Available Potassium
- Weigh 2.5 g of 2 mm air-dry soil (0.1 g accuracy) into a 150 ml Erlenmeyer flask.
- Add 25 ml of extracting solution (soil to solution ratio 1:10).
- Stopper and shake the suspension for exactly 5 minutes on reciprocating shaker (180 + oscillations/min). Immediately filter it.

**Calculation**
Available K ppm = \( \frac{\text{Reading (ppm)} \times 25}{2.5} \)

- 0.2 N acetic acid, 0.25 N ammonium nitrate, 0.013 N HNO$_3$, 0.015 N NH$_4$F, 0.001M EDTA
- Potassium chloride standard solution: Make a stock solution of 1000 ppm K by dissolving 1.908 of AR grade potassium chloride in distilled water and diluting up to 1 litre. Prepared 100 ppm standard by diluting 100 ml of 1000 ppm stock solution to one litre with the extracting solution.
- Standard curve: Pipette 1, 5, 10, 15, 20 and 30ml of 100 ppm solution into 100 ml volumetric flasks and bring the volume to mark with extracting solution. The solutions contain 1, 5, 10, 15, 20 and 30 ppm K, respectively.

3.6.5.2.5. Determination of Organic Carbon
Organic carbon was estimated by method of Dutta *et al.* (1962). Oxidation of soil organic matter is carried out by dichromate-sulphuric acid mixture and the intensity of the colour
of the chromium sulphate formed is measured to give directly the amount of carbon oxidized.

1. Take one gram of soil (passed through 0.2 mm sieve) in a dry 250 ml conical flask.
2. Add 10 ml of 1N potassium dichromate and swirled by 20 ml of conc. sulphuric acid and swirled again and keep for 30 min on an asbestos sheet.
3. Prepare a blank by pipetting 10.0 ml of 1.0 N potassium dichromate solution into an empty 250 ml Erlenmeyer flask.
4. Immediately use a graduated cylinder to add 100 ml of de-ionized water to each flask. Swirl briskly to mix.
5. Set the wavelength 610 nm and auto zero by placing reference standard. Place the test sample and take the reading.

Reagents
- 1N potassium dichromate (49.04g l⁻¹)
- Concentrated sulphuric acid (sp. grade 1.84)
- Sucrose (AR grade)

Preparation of Standard Calibration Curve
Take 1-25 mg of anhydrous sucrose (AR) in 100 ml dry conical flasks. Add 10 ml of 1 N K₂Cr₂O₇ solution and 20 ml of concentrated H₂SO₄ to each conical flask. The flasks are swirled a little and after 10 min add 100 ml distilled water. Thereafter, readings are taken in the spectrophotometer at 610 nm after adjusting the blank to zero. A standard curve is drawn plotting the concentrations of carbon (in sucrose) on X axis and colorimeter readings (R) or Y axis. The concentration of carbon in sucrose was calculated as follows.

Calculation of concentration of carbon from sucrose for standard curve:

<table>
<thead>
<tr>
<th>Quantity of sucrose (mg)</th>
<th>Percent carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>1</td>
<td>0.042</td>
</tr>
<tr>
<td>2</td>
<td>0.084</td>
</tr>
<tr>
<td>4</td>
<td>0.168</td>
</tr>
<tr>
<td>6</td>
<td>0.262</td>
</tr>
<tr>
<td>8</td>
<td>0.336</td>
</tr>
<tr>
<td>10</td>
<td>0.42</td>
</tr>
<tr>
<td>12</td>
<td>0.504</td>
</tr>
<tr>
<td>14</td>
<td>0.588</td>
</tr>
<tr>
<td>16</td>
<td>0.672</td>
</tr>
<tr>
<td>18</td>
<td>0.756</td>
</tr>
<tr>
<td>20</td>
<td>0.840</td>
</tr>
<tr>
<td>25</td>
<td>1.05</td>
</tr>
</tbody>
</table>
Organic carbon (%) was calculated from the standard curve with the help of colorimetric reading of the test sample.

3.6.5.3 Volumetric Soil Moisture Content (θ) and Electrical conductivity of Soil (ECw Soil)

θ and ECw soil was measured by HH2 moisture meter with W.E.T sensors (Delta-T Devices Ltd. Cambridge, UK). The measurement of soil moisture was made by dipping the sensor about 3 cm around the rhizosphere of plants. Both the parameters were recorded and five replications of each parameter were taken during all the assigned days of water stress and well irrigated controls.

3.6.5.4. Relative Water Content

Leaf relative water content (RWC) was measured at 0, 3, 6, 12 and 15 days of water withholding (DOWW) in WT and CsTLP transgenic lines (TL1 and TL2) according to Barrs and Weatherley (1962). Fully expanded terminal leaflets of 2nd and 3rd leaves from apex were excised and fresh weight (FW) was immediately recorded in between 9 am and 10 am; then the leaves were soaked for 12 h in distilled water at room temperature under constant light, and the turgid weight (TW) was recorded. After drying for 48 h at 60°C total dry weight (DW) was recorded. RWC was calculated according to following equation:

\[
RWC (\%) = \left\{ \frac{(FW-DW)}{(TW-DW)} \right\} \times 100
\]

3.6.5.5. Measurement of Stomatal Conductance (gS) and Photosynthetic Activity (PN)

Stomatal conductance (gS) and net photosynthetic rate (PN) data were monitored on the second terminal leaflets of second leaf with a portable photosynthesis system LI-6400 (Li-COR, Lincoln, NE, USA). Infrared gas analyzers were zeroed for CO2 and H2O using a CO2 scrubber and desiccant, respectively, before calibrating span for CO2 and H2O. Span for CO2 and H2O was adjusted using a calibrated gas (505 cm³ m⁻³; Li-COR) and a portable dew point generator (LI-610; Li-COR), respectively. All observations were recorded at 1000 μmol m⁻² s⁻¹ using a cool light source (6400-02 LED) fitted on top of the leaf chamber, and CO2 concentration of 400 cm³ m⁻³ using a 6400-01 CO2 injector. A time interval of 90 s was given for leaf to equilibrate to the new conditions in each measurement.
3.6.5.6. Measurement of Chlorophyll Fluorescence ($F_v/F_m$)

Plant samples have been dark-adapted overnight before measurements using the clips (provided by the manufacturer). Chlorophyll fluorescence was measured using the same photosynthesis system (LI-6400; Li-COR, Lincoln, NE, USA) with an integrated fluorescence chamber head (LI-6400–40). The leaves were detected repeatedly in the gas exchange measurement. The experiment was made at a leaf temperature of 30°C. The portable measuring system automatically determines the $F_v/F_m$ by providing weakly modulated light < 0.1 μmol m$^{-2}$s$^{-1}$ and 600-ms saturating flash (7200 μmol m$^{-2}$s) for one second.

3.6.5.7. Estimation of Chlorophyll content

Chlorophyll was isolated and estimated following the method described in Section 3.2.2.2.

3.6.5.8. Leaf Proline Content

The method of Bates et al. (1973) was used to measure the proline in the leaves of control and stressed plants.

- Frozen leaves (500 mg) were homogenized in 10 ml of (3%) aqueous solution of sulfosalicylic acid and filtered.
- An aliquot of 2 ml filtrate was mixed with 2ml of ninhydrin and 2 ml of glacial acetic acid and incubated at 100°C for one hour. Finally, the reaction was completed in an ice bath.
- Thereafter, 4 ml of toluene was added and contents of the tube were inverted during 20 seconds. After this, toluene phase was removed and allowed it to stabilize. Finally, its absorbance was determined at 520 nm using a visible light spectrophotometer (Shimadzu, Japan). Proline concentration was determined following a calibration curve prepared using concentrations range of 0-3 μg μl$^{-1}$ proline (Sigma-Aldrich, Bangalore, India). The concentration of proline was determined against a standard curve and calculated by adopting the following equation:

$$\text{Proline (μ mole g}^{-1}\text{ leaf tissue)} = [\text{(μg of proline ml}^{-1}\text{ from the curve} \times \text{volume of toluene}/115.5 \text{ μg μmole}^{-1})] \times \text{[sample (g)/5]}$$
3.6.5.9. Amylolytic Activity on Starch Agar Plate

Amylolytic activity was performed as per the procedure mentioned below:

3.6.5.9.1. Sample Preparation

- Leaf tissues from 3, 6, 9, 12 and 15 day of water stress duration as well as control well irrigated plant samples were homogenized in chilled five volumes of 0.2 M Na-acetate buffer (pH 6.0) containing 3 mM CaCl$_2$ (Li et al. 1992).
- Homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant of all the samples were collected and filter sterilized individually and aliquoted to 1.5 ml sterile eppendorfs and stored at -20°C. As a control, 500 µl of each of the filter sterilized sample was autoclaved and stored at -20 °C.
- In addition, 0.2 M Na-acetate buffer (pH 6.0) was also filter sterilized, aliquoted and stored at -20 °C and served as buffer control.

3.6.5.9.2. Amylolytic Activity

- Starch agar media was autoclaved, cooled to 45°C and poured into autoclaved Petri-plates.
- After medium solidification, uniform size wells around the periphery of starch agar petriplate were created.
- Pre-frozen supernatant (100 µl) of each of water day stress sample along with controls were poured separately into wells created on starch agar plate and Petri-plates were kept overnight in an incubator at 37 °C.
- After overnight incubation, Petri-plates were flooded with iodine solution (0.2%) for 5 minutes. Excess iodine solution was poured off and the plates were examined for starch hydrolysis in terms of clear zone around the wells.

3.6.5.10. Leaf Carbohydrate Analysis

Starch, Total Soluble Sugar (TSS) and Reducing Sugars (RS) were estimated in 100 mg (dwt.) samples. For this, the samples were extracted in 80% ethanol and were centrifuged at 10,000 rpm for 10 min. While the resultant supernatant was used for TSS and RS, the residual precipitate was dried and hydrolyzed with Perchloric acid (52%) for the estimation of starch as liberated glucose using the anthraone reagent method of Adams et al. (1980).
The methods used included the Phenol sulphuric acid method of Dubios et al. (1956) from reducing sugars.

### 3.5.6. Evaluation of Transgenic Tubers for Food Prospects

The tubers of WT and CsTLP transgenic line, TL1, were sown in pots in contained facility as described in Section 3.5.2.1. After emergence, the plants were irrigated regularly on a 7 day regime. Tuber harvested from CsTLP transgenic lines (TL1) were analyzed for color, texture, micro-structural features and storage attributes useful for food industry and were compared with that of the WT tubers.

#### 3.6.6.1. Color and Textural Analysis Characteristics

Color measurements of the tubers from WT and CsTLP line 1 tubers were carried out in triplicate using a Hunter colorimeter model D25 optical sensor (Hunter Associates Laboratory Inc., Reston, VA, USA) on the basis of $L^*$, $a^*$ and $b^*$. The instrument ($45^\circ/0^\circ$ geometry, $10^\circ$ observer) was calibrated against a standard red colored reference tile ($L^* = 25.80$, $a^* = 29.25$, $b^* = 12.11$). Three tubers from each cultivar were washed and their periderm was peeled with a knife. The peeled tubers were split into halves and Hunter $L^*$, $a^*$ and $b^*$ values at the cleaved surface of the potato tubers were measured. The peeled and cut potatoes from each cultivar were evaluated for the development of discoloration after 30 min. The changes in $L^*$, $a^*$ and $b^*$ values during 30 and 60 min storage ($\Delta L = L_{ST} - L_0$, $\Delta a = a_{ST} - a_0$ and $\Delta b = b_{ST} - b_0$, where $L_0$, $a_0$ and $b_0$ represent the readings at time zero, and $L_{ST}$, $a_{ST}$ and $b_{ST}$ represent the individual readings after storage associated with enzymatic browning were calculated from the data.

The total color difference ($\Delta E$) was evaluated using $\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$

Texture profile analysis (TPA) of the tubers was performed using TA.XT plus Texture Analyzer (Stable Micro Systems, England). A single tuber was placed at the center of heavy-duty platform (HDP/90) and subjected to penetration with a 2 mm diameter probe (P2) at a pre-test, test and post-test speed of 1 mm/s, up to a distance of 10 mm in two cycles using a 5 kg load cell. Bioyield point of the tubers was determined. The tubers were then cut into cubes of each side of 10 cm and compressed with a 40 mm diameter aluminum cylinder probe (P/40) at a pre-test, test and post-test speed of 1 mm s$^{-1}$, up to 95
% compression. Five replications were carried out and the textural parameters of hardness, springiness, cohesiveness, chewiness and adhesiveness were calculated as described by Bourne (1978).

3.6.6.2. Morphological Properties of Starch granules

For determining the shape of the starch granules the potato tubers of WT and TL1 plants were crushed in liquid nitrogen and lyophilized (MAXI-Drylyo). The samples were loaded on double sided carbon tape or aluminium stub and coated with carbon sputter coating unit (E1010, Hitachi). Scanning electron micrographs of starch samples were captured by SEM (S3400N, Hitachi, Japan) at different magnifications.

3.6.6.3. Storage Attributes of Tubers

Harvested tubers of WT and TL1 plants were cleaned by giving a series of washing with tap water to remove any dirt particles adhered with the surface of tubers. This step was followed by shade drying for 48 hours at RT. All the tubers were stored in cold store under dark conditions at 4°C for 3 months and were accessed for sprouting and textural analysis.

3.7. Production of CsPPO Transgenic Plants and Molecular Characterization of CsPPO

Transgenic plants were raised according to the standardized transformation procedure described in Section 3.4.2

3.7.1. Antibiotic Selection

Putative transformants harboring CsPPO were screened on plant selection media according to method followed in Section 3.4.2.1.1.

3.7.2. Molecular Characterization of Transgenic CsPPO Plants

3.7.2.1. PCR Confirmation

DNA was isolated from the putative CsPPO transgenic plants following the protocol described in Section 3.4.3.2.1 and the integration of CsPPO in transgenic potato was confirmed by using gene specific primer pair and PCR conditions mentioned in Tables 3.1 and 3.3.
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3.7.2.2. Slot Blot Analysis

Slot blot analysis was performed to further check the integration of CsPPO gene in transgenic CsPPO plants following the procedure mentioned earlier in Section 3.4.3.4.

3.7.2.3. Semi-Quantitative PCR Analysis

RNA isolation and semi quantitative PCR analysis for CsPPO expression was performed according to method followed in Section 3.4.4.1.3.

RNA isolation and cDNA synthesis was proceeded following the steps mentioned in Section 3.2.4.2 and 3.2.4.3. Equal amount of synthesized cDNA of WT and transgenic plants were used as a template for PCR using CsPPO specific expression primers and PCR conditions mentioned in Table 3.1 and Table 3.3. The expression of 26S rRNA gene by 26S rRNA based primers and conditions mentioned in section were used as internal control for expression studies. The amplified products were checked on 1.2% (w/v) agarose gel as described earlier.

3.7.3. Phenotypic Analysis of Transgenic Plants

The plants of WT and CsPPO transgenic line (TP), verified molecularly were multiplied under in vitro conditions for phenotypic analysis. Phenotypic analyses of transgenics relative to WT were performed by studying any differences in the shape of microtubers, growth of plants under in vivo conditions and tuber characteristics after harvesting as described earlier in Section 3.5.

3.7.4. Determination of Specific PPO Activity

Specific PPO activity of in vitro leaves and stem portions of WT and transgenic PPO plants was determined by the following procedure:

1. Crush 1 gm of plant sample in liquid nitrogen. Crush to make fine powder. Add to it 80% chilled acetone and let it thaw and grind it.
2. Centrifuge it at 12000 rpm at 4°C for 15 min.
3. Throw the supernatant and again add 7-10 ml 80% chilled acetone. Dispense the pellet and again centrifuge. Repeat the step until whole pellet became white in color.
4. Give final washing with 100% chilled acetone by dispersing the pellet. Centrifuge at 15000 rpm for 15 min at 4°C.
5. Decant the supernatant and vacuum dry the pellet for 30 min. The dry powder was stored at -20°C.

6. Acetone powder (20 mg) along with some amount of PVPP was taken in mortar. Grind it with liquid N₂. Add liq. N₂ prior to the addition of above two compounds.

7. Add 750 μl of ice-cold buffer containing 0.2 M potassium phosphate buffer (pH 7.0), 0.35 M KCl, 0.5% Triton X 100 and 50 μl of 75 mg PMSF. Grind it with thawing.

8. The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C.

9. Supernatant was take and stored at -20°C.

3.7.4.1. Enzyme Assay

PPO was estimated according to the method of Moore and Flurkey (1990). PPO activity must be recorded at 28°C. For this, set the blank with citrate buffer (850 μl) and catechol (100 μl) and extraction buffer potassium phosphate (50 μl). Take reading by adding the following components: catechol (100 μl), citrate buffer (850 μl), enzyme (50 μl). Shake the cuvette and a change in absorbance at 410 nm was monitored for 3 min and the velocity of the reaction was determined from the linear portion of the curve.

3.7.5. Minituber Production

Minitubers from WT and transgenic PPO plants (TP) were produced as per the procedure mentioned in Section 3.3.6.