Chapter 4

RNAi Mediated Silencing of IPK1 gene to reduce phytic acid
4.1 INTRODUCTION

It is evident from the results discussed in previous chapter that myo-inositol-3-phosphate synthase (MIPS, EC 5.5.1.4) enzyme catalyzes the first step of phytic acid biosynthesis in the developing rice seed (Yoshida et al., 1999). The downregulation of MIPS brings about a substantial reduction in the level of phytate in transgenic seeds. However, the inadvertent change revealed in seed myo-inositol contents might have a negative impact on plant inositol metabolism, as myo-inositol-3-phosphate, the product of the MIPS, is the only known precursor for the de novo synthesis of myo-inositol (Keller et al., 1998; Majumder et al., 2003; Panzeri et al., 2011). Hence in order to reduce phytate content in seeds without disturbing the related pathways, late pathway enzymes involved in phytic acid biosynthesis (IPK's) in rice could be targeted and effects could be analyzed.

The inositol phosphate kinase is believed to catalyze the final step in phytic acid biosynthesis, whereby the InsP₅ molecule is phosphorylated at the 2ⁿᵈ position (Stephens and Irvine, 1990; Brearley and Hanke, 1996; York et al., 1999). The InsP₆ biosynthetic pathway was previously described in Saccharomyces cerevisiae (York et al., 1999) and the pathway shared a common final step with that in Dictyostelium discoideum of the phosphorylation of Ins(1,3,4,5,6)P₅ to InsP₆ by a 2-kinase enzyme designated as IPK1 (EC 2.7.1.158). The S. cerevisiae IPK1Δ mutant showed an almost complete inability to synthesize InsP₆, and showed a decrease in the ability to export mRNA from the nucleus. Since then several myo-inositol kinase enzymes have also been identified in plants including myo-inositol kinase (Shi et al., 2005), Ins(1,3,4)P₃/5/6-kinase (Wilson and Majerus, 1997), Ins(1,4,5)P₃/6/3/5-kinase (Stevenson-Paulik et al., 2002), and Ins(1,3,4,5,6)P₅ 2-kinase (Stevenson-Paulik et al., 2002; Sweetman et al., 2006). Recently, Stevenson-Paulik et al., (2005) examined the Ins(1,4,5)P₃/6/3/5-kinase gene (Atlpk2β-1) and the Ins(1,3,4,5,6)P₅ 2-kinase gene (Atlpk1-1) using T-DNA insertion mutants in Arabidopsis. Phytate was reduced in the Atlpk2β-1 mutant by 35%, in the Atlpk1-1 by 83%, and in the double mutant by greater than 95%.

In the present study we generated transgenic rice plants by silencing the last step of phytic acid biosynthesis in indica rice cultivar, by manipulating the gene expression of IPK1 through the use of seed specific promoter, Ole18 and Ltp2 by RNAi mediated approach. The
resulting transgenics were analyzed at the molecular and biochemical level, which revealed reduction in phytate levels, along with an increase in the amount of available phosphorus. In addition, we also estimated the change in concentration of different metals in milled rice grains, which may be affected, due to reduction of phytate levels in seeds. Different agronomic traits of the transgenics were also analyzed and compared with the non-transgenic rice plants.

### 4.2 MATERIALS

#### 4.2.1 Plant materials and growth conditions

*Oryza sativa* L. Subspecies *indica* cv. Swarna procured from Chinsurah Rice Research Station, Hooghly, West-Bengal, were used for cloning purposes. Rice seeds were germinated following the procedure mentioned in section 3.2.1. For genetic transformation purpose *Oryza sativa* L. Subspecies *indica* cv. Pusa Sugandhi II was available from IARI, ICAR, India.

#### 4.2.2 Vectors and bacterial strains used

The vectors and bacterial strains used were same as mentioned in section 3.2.2.

#### 4.2.3 Molecular biology kits used

All the kits used for molecular biology were same as mentioned in section 3.2.3.

### 4.3 METHODS

#### 4.3.1 Amplification of *IPK1* gene from rice

#### 4.3.1.1 Isolation of RNA and its quantification

Seeds of *indica* rice cultivar Swarna were germinated and RNA was isolated and quantified as per the procedure elucidated in section 3.3.1.1.
4.3.1.2 cDNA synthesis and RT-PCR reaction

cDNA was synthesized from 5 μg of DNasel treated purified RNA using the Superscript III reverse transcriptase two step RT-PCR kit (Invitrogen, USA) following manufacturer’s protocol (as mentioned in section 2.3.3.2). After synthesis of cDNA, PCR reaction was set up to clone IPK1 (Gene bank accession no.- AK102842) gene using Pfx polymerase (Invitrogen, USA) and gene specific primer pair. The primer sequences used are as follows:

**IPK1 F:** 5'-CTGCTCTTCTAATTTCGACC-3'

**IPK1 R:** 5'-CTTCTTAATGTTTGTCTACTG-3'

PCR amplifications were performed in a volume of 50 μl reaction mixture set up as mentioned below

<table>
<thead>
<tr>
<th>Components (Concentration)</th>
<th>Volume used per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Taq buffer without MgSO(_4) (Invitrogen)</td>
<td>5 μl</td>
</tr>
<tr>
<td>50mM MgSO(_4) (Invitrogen)</td>
<td>3.5 μl</td>
</tr>
<tr>
<td>10mM dNTPs (Roche)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Primers (100ng/μl) Forward and reverse</td>
<td>1 μl each</td>
</tr>
<tr>
<td>cDNA template (200ng/ μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td><em>Pfx Taq</em> Polymerase (2.5U/ μl)</td>
<td>0.4 μl</td>
</tr>
<tr>
<td>Sterile dH(_2)O</td>
<td>36.1 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 μl</strong></td>
</tr>
</tbody>
</table>

Amplification was carried out in a thermocycler (Eppendorf, Germany). The PCR was programmed for 5 min at 95°C followed by 35 cycles of 1 min at 94°C; 1 min at 54°C and 1 min at 68°C with last step of additional 10 min extension at 68°C. PCR products were then separated by agarose (1%) gel electrophoresis and visualized with ethidium bromide and photographed using Gel Documentation XR system (BIO-RAD) and analyzed through QuantityOne software system.
4.3.2 Cloning of *IPK1* gene in entry vector pENTR™/ D-TOPO

The *IPK1* PCR product obtained was further purified and cloned into pENTR™/ D-TOPO (Invitrogen) vector as per the procedure described in section 3.3.2. The clones obtained were checked and confirmed by PCR based analysis using gene specific primer pair.

4.3.3 Sequencing of *IPK1* gene

The pENTR-*IPK1* clones after PCR confirmation were sequenced using gene specific primers at Chromous Biotech Ltd. Company. The sequence homology and identity was analyzed through free software available viz. BLAST analysis (www.ncbi.org). The probable protein homology was studied through BLASTP analysis.

4.3.4 Vector construction for Bacterial expression analysis

For expression analysis of the cloned *IPK1* gene, pRSET-A vector and *E.coli* BL21 strain (Invitrogen, USA) were used. The cloned 1.1 Kb (*IPK1*) fragment was excised from pENTR-D-TOPO vector through *XhoI* / *PstI* restriction enzymes (Roche, Switzerland) digestion as detailed below.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>17 µl</td>
</tr>
<tr>
<td>10X Restriction Assay buffer (Roche)</td>
<td>3 µl</td>
</tr>
<tr>
<td>pENTR-<em>IPK1</em> plasmid DNA (500ng µl(^{-1}))</td>
<td>5 µl</td>
</tr>
<tr>
<td>RNAseA (10mg ml(^{-1}), Sigma-Aldrich)</td>
<td>3 µl</td>
</tr>
<tr>
<td>Restriction enzyme (10U µl(^{-1}) <em>XhoI</em>)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Restriction enzyme (10U µl(^{-1}) <em>PstI</em>)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Final volume</td>
<td>30 µl</td>
</tr>
</tbody>
</table>

The excised fragments were then purified and ligated into pRSET-A vector using T\(_4\) DNA ligase (Fermentas) and then transformed into competent cells of *E.coli* strain DH5α by Calcium chloride method (as mentioned in section 2.3.1.6) (Sambrook and Russel, 2001). The recombinant plasmid was selected against ampicillin antibiotic and after confirmation
through restriction digestion; the pRSET-IPK1 vector was then transformed into *E.coli* BL21 cells.

### 4.3.5 Expression analysis of IPK1 gene in bacterial system

The recombinant *E.coli* BL21 cells harboring pRSET-IPK1 vector were grown at 25°C for 16 hrs in presence of different concentration (0.3 mM, 0.5mM and 1mM) of IPTG solution (Sigma-Aldrich). After standardization of optimum IPTG concentration for induction of IPK1 protein, the total protein was isolated from bacterial cells in native condition following the manufacturer’s protocol (Invitrogen). The total protein was isolated from 50 ml of IPTG induced bacterial culture and further purified following the procedure described in section 3.3.5.1.

#### 4.3.5.1 Western Blot analysis of purified protein

Western blot analysis was performed to analyze the expression of IPK1 protein product (Sambrook and Russell, 2001). The analysis was carried out according to the protocol mentioned earlier in section 3.3.5.2.

### 4.3.6 RNAi vector construction

#### 4.3.6.1 Cloning of IPK1 gene in knockdown vector pIPKb006

The *IPK1* gene cloned in the entry vector pENTR™/ D-TOPO was subcloned into the destination vector pIPKb006 (IPK, Gatersleben, Germany) (Himmelbach *et al.*, 2007) using LR clonase (Invitrogen, USA) based Gateway recombination system following the procedure mentioned in section 3.3.6.1. The streptomycin resistant colonies harboring pIPK1-006 recombinant plasmid were further screened and confirmed by PCR reaction using *IPK1* gene specific primers.
4.3.6.2 Cloning of promoters upstream of IPK1 gene in pIPK1-006 vector

The tissue specific promoters Ole18 and Ltp2 cloned in TOPO vectors were digested with restriction endonuclease enzymes SpeI and HindIII and further subcloned in the pIPK1-006 vector following the procedure already mentioned in section 3.3.6.2. The recombinant plasmid clones of pOle18-IPK1-006 and pLtp2-IPK1-006 were confirmed by PCR based analysis using specific primers and also by restriction digestion mediated by endonuclease enzyme SpeI and HindIII. The complete RNAi vectors pOle18-IPK1-006 and pLtp2-IPK1-006 were used for rice transformation separately.

4.3.7 Maintenance of the recombinant vector constructs

The bacterial cells of E. coli BL21 harboring pRSET-fRKT and E. coli DH5α harboring the recombinant plasmids (pOle18-IPK1-006 and pLtp2-IPK1-006) were maintained as glycerol stocks and stored in -80°C freezer until further use.

4.3.8 Tissue culture and genetic transformation

The preparation of media and culture conditions were similar to the one elucidated in section 2.3.2.1 and 2.3.2.2. Biolistic method of transformation was same as described in section 2.3.2.3. The immature and mature embryos of indica rice cultivar Pusa Sugandhi II were bombarded with the prepared plant transformation vector constructs (pOle18-IPK1-006 and pLtp2-IPK1-006) using Particle Delivery System (PDS-1000/He system, BIORAD, Hercules, CA, USA) following manufacturer's instruction. Following bombardment, the embryos were transferred to the callus induction medium and after proper selection and regeneration as described earlier in section 2.3.2.4, the regenerated plantlets were transferred to rooting medium (MS without hormone). After development of proper root system, the putative transgenics were grown in greenhouse (Dept. of Botany, CU) to maturity. All the transgenic plants obtained were fertile with normal phenotype. The T0 transgenic rice plants grown in greenhouse were confirmed for the presence of transgene. Subsequently the selected T0 transgenic plants with normal phenotype were derived and grown up to T3 and
T₄ generation. Further molecular and biochemical analysis were performed with the T₂, T₃ transgenic plants and T₃, T₄ transgenic seeds obtained.

4.3.9 Molecular analysis of transgenics

4.3.9.1 Screening of transgenic plants by PCR

The integration of RNAi vector constructs (pOle18-IPK₁-006 and pLtp2-IPK₁-006) in subsequent generations of transgenics (T₀-T₄) were confirmed through genomic PCR, using genomic DNA extracted from the transgenic plants following modified rapid DNA isolation protocol (Huang et al., 1997) as described in section 2.3.3.1. The PCR amplification was performed with 150-200 ng of genomic DNA, using RGA₂ intron specific primer pair. The primers used and the reaction set up was same as mentioned in section 3.3.9.1.

4.3.9.2 Southern hybridization of transgenic plants

Southern hybridization analysis was performed with PCR positive transgenic plants of T₃ and T₄ generation to confirm the stable integration of the transgene following the method described in Sambrook and Russel (2001). The complete procedure for Southern blot analysis followed was same as described in section 3.3.9.2. Genomic DNA (10 μg) was digested with EcoRI and HindIII (Fermentas), separated on a 1% agarose gel and transferred to a nylon membrane (Hybond N⁺, Amersham, GE Healthcare, USA). Hybridization was carried out using the RGA₂ intron from wheat (present in the vector pOle18-IPK₁-006 and pLtp2-IPK₁-006), labeled with α-P³² dCTP radioisotope (BARC, India), using Decalabel DNA labeling kit (Fermentas, USA) according to the manufacturer’s instructions.

4.3.9.3 Transgene expression analysis

Total RNA was isolated from the mature dehusked T₃/T₄ seeds (100 mg) by modified RNA isolation protocol (Meng and Feldman, 2010). Total RNA was quantified and cDNA was synthesized using the Superscript III reverse transcriptase, two step RT-PCR kit (Invitrogen,
USA). The entire protocol followed was same as described in section 2.3.3.2. Subsequently, the cDNA was used as template for RT-PCR reaction using IPK1 gene specific primer pairs.

**Semi-quantitative RT PCR analysis**

After synthesis of cDNA, RT-PCR reaction was set up using IPK1 gene specific primers and the set up was as detailed below.

<table>
<thead>
<tr>
<th>Components (Concentration)</th>
<th>Volume used per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Assay buffer with MgCl2 (Chromous Biotech)</td>
<td>5 µl</td>
</tr>
<tr>
<td>25mM MgCl2 (Chromous Biotech)</td>
<td>4 µl</td>
</tr>
<tr>
<td>10mM dNTPs (Roche)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Primers (100ng/µl) Forward and reverse</td>
<td>1 µl each</td>
</tr>
<tr>
<td>cDNA template (200ng µl⁻¹)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq Polymerase (3U µl⁻¹)</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>35.7µl</td>
</tr>
<tr>
<td>Final volume</td>
<td>50µl</td>
</tr>
</tbody>
</table>

The PCR programming and other conditions were same as mentioned earlier in section 4.3.1.2. Besides IPK1, PCR reaction was also set up for amplification of house-keeping gene β-tubulin which was used as an internal control. The specific primers used and other details were same as described in section 3.3.9.2. The PCR products obtained were then separated by agarose (1%) gel electrophoresis and visualized with ethidium bromide and photographed using Gel Documentation XR system (BIO-RAD).

**Quantitative RT-PCR (qPCR) analysis**

The qRT-PCR reaction using SYBR Green (Fermentas) was performed with gene specific primers detailed below.

**InIPK1 F:** 5'-TGAGAAGATTGTCAGGGACTTC-3'

**InIPK1 R:** 5'-CGTACTCAGAATCTGTTGTTCCA-3'
The PCR cycle was as follows: 95°C for 1 min, 59.6°C for 1 min and 72°C for 1 min. The procedure was according to the manufacturer's instructions (CFX 96 Real time system, BioRad). The quantitative variation between different samples was evaluated by the ΔΔCt method, and the amplification of β tubulin gene was used as internal control to normalize all data. To validate the results, each experiment was performed in replicates.

4.3.10 Biochemical analysis of transgenic rice

All the biochemical analyses performed were same as detailed in section 3.3.10 in chapter 3.

4.3.11 Morphological analysis of transgenic rice

All the morphological analyses viz. seed viability, histology of embryo, seed germination assay (CGT and AAT) and agronomic study of transgenics were same as mentioned in section 3.3.11 in chapter 3.

4.3.12 Introgression of low phytate traits into local cultivar

Low phytate transgenic rice plants (IO6-97-9-4, grown under greenhouse conditions) were hybridized with the local rice cultivar Swarna. The hybridization was performed as per the procedure described in section 3.3.12. The resultant hybridized seeds obtained were germinated and further grown under greenhouse conditions along with the non-hybridized control Swarna plants. The resulting plants were screened with RGA2 intron specific primer pairs for the presence of the transgene. The positive hybridized plants obtained were grown to maturity and again backcrossed with the parental Swarna plants. The resulting BC₁ F₁ seeds have been collected and will be backcrossed further to obtain near isogenic lines.

4.3.13 Statistical analysis

All statistical analysis was performed using the Graph Pad Prism 5 software. The experimental data were recorded taking mean value from three independent series, each
done with three replicates, and the results presented as means ± standard error (SE), based on three replications. The statistical significance at P ≤ 0.05 has also been calculated.

4.4 RESULTS

4.4.1 RNAi vector construction- IPK1 under the control of tissue specific promoters

In the present study knockdown vector pIPKb006 was used in order to mediate efficient cloning of targeted gene based on gateway recombination (Himmelbach et al., 2007). After isolation of RNA from swarna (Fig. 48a), IPK1 gene was amplified using gene specific primer pairs (Fig. 48b). The PCR product of IPK1 obtained was cloned in entry vector pENTR™/ D-TOPO (Fig. 49a & 49b) followed by subcloning into knockdown vector pIPKb006 by gateway based recombination system.

Figure 48: Cloning of IPK1 gene from indica rice cultivar Swarna. (a) RNA isolated from indica rice cultivar Swarna. (b) Amplification of IPK1 gene using gene specific primers from cDNA synthesized from isolated RNA. (M= λ DNA marker, EcoRI / HindIII digest; Lane 1-3= aliquots of RNA isolated from Swarna and subsequent amplification of IPK1 from each aliquot).
Figure 49: Cloning of *IPK1* gene in pENTR™/ D-TOPO vector. (a) Vector diagram showing *IPK1* gene cloned into pENTR™/ D-TOPO vector. (b) PCR and restriction confirmation of pENTR-*IPK1* vector constructs (M= 1Kb gene ruler; Lane 1= Restriction confirmation of pENTR-*IPK1* vector construct; Lane2= PCR confirmation of pENTR-*IPK1* vector showing 1.1 Kb fragment).

Further, the tissue specific promoters (*Ole18* and *Ltp2*) were subcloned upstream of the *IPK1* gene separately at the *SpeI* and *HindIII* site of the MCS in the knockdown vector. The complete vector constructs (pOle18-*IPK1*-006 and pLtp2-*IPK1*-006) (Fig. 50) were then confirmed by restriction digestion with endonuclease *EcoRI* which is present in the vector backbone as well as the *IPK1* gene itself (*Ltp2* promoter also has one *EcoRI* site) (Fig. 51).

Figure 50: Vector map of RNAi vector constructs pOle18/Ltp2-*IPK1*-006 showing the complete transgene cassette.
Figure 51: RNAi vector construction with \textit{IPK1} gene for biolistic transformation. (a) Plasmid DNA of complete vector constructs \textit{pOle18-IPK1-006} and \textit{pLtp2-IPK1-006}. (b) Restriction confirmation of complete vector constructs with \textit{EcoRI} (IO6 shows three bands and IL6 shows four because \textit{Ltp2} also has one \textit{EcoRI} site) digestion showing desirable bands. (M= \textit{λ} DNA marker, \textit{EcoRI} / \textit{HindIII} digest and 1 kb gene ruler).

4.4.2 Sequencing of cloned gene

The \textit{IPK1} gene cloned in pENTR\textsuperscript{TM}/D-TOPO vector were sequenced by using gene specific primers. Analysis of the sequence data obtained was carried out through free software available viz. BLASTN (www.ncbi.org). The analysis of gene sequences revealed that \textit{IPK1} shows 100% homology with the reported sequences of \textit{IPK1} (Gene Bank accession no.-AK102842) (Fig. 52).
RNAi mediated silencing of IPK1 gene to reduce phytic acid
4.4.3 Bacterial expression analysis of IPK1 gene

To confirm the functional activities of the putative IPK1 gene, cDNAs were cloned into the bacterial expression vector pRSET-A (Fig. 53a) in frame with N-terminal His tags. The recombinant plasmids were confirmed by subsequent restriction digestion and PCR analysis (Fig. 53b). Following induction in the E. coli BL21 strain with 0.5 mM IPTG (Fig. 54a) and purification with Ni-NTA resin (Fig. 54b), protein expression was confirmed by Western-blot analysis (Fig. 54c) using both anti-HisG antibodies.

Figure 53: Bacterial expression analysis of IPK1 gene. (a) Vector diagram of pRSET-IPK1. (b) PCR (Lane 1 and 2) and restriction confirmation (Lane 3 and 4) of the pRSET-IPK1 vector. (M= 1kb gene ruler).
4.4 Generation of transgenic rice plants

The key to genetic-engineering strategies is introduction of desirable gene or set of genes into the genomes of the target plant, by different methods of transformation. Biolistic method of transformation is a well-known phenomenon, which we have utilized in our study for generation of stable transgenics. After bombardment, the embryos showed callus initiation within one week. The embryogenic calli were relatively dry, milky white in colour (Fig. 55a). After successive selection in presence of hygromycin B, only few embryogenic calli survived, which were transferred to the regeneration media (Fig. 55b). The regenerated plants were further grown on rooting medium and finally in greenhouse till maturity (Fig. 55c & 55d). All the plants with normal phenotype were selected and the plants were fertile.
Figure 55: Genetic transformation of *indica* rice cultivar with *IPK1* constructs. (a) Transformed calli proliferating in MS media supplemented with hygromycin. (b) Regeneration of putative transgenics on regeneration media. (c) Putative transgenics grown in Yoshida nutrient solution for development of proper root system. (d) Transgenic rice grown in greenhouse (containment facility) at Chinsurah Rice Research Station, West Bengal.
4.4.5 Molecular analysis of transgenic rice

4.4.5.1 Screening of transgenics

PCR confirmation from genomic DNA of the subsequent generation (T₀-T₄) of transgenics showed the integration of transgene cassette as amplified by intron specific primers (RGA2 intron). The wheat RGA2 intron (310 bp) present in the RNAi vector was amplified from transgenics, however it was absent in the non-transgenic control. Plasmid DNA of pOle18-IPK1-006 and pLtp2-IPK1-006 were used as template for positive control reactions (Fig. 56).

![Figure 56: Screening of IPK1 downregulated transgenic rice by genomic PCR analysis.](image)

Amplification of RGA2 intron (310bp) confirms integration of the transgene cassette in transgenic plants expressing (a) pOle18-IPK1-006 and (b) pLtp2-IPK1-006. (M= 1 Kb gene ruler, NT= Non-transgenic control, P= positive control, 1-22= different transgenic lines expressing IPK1 gene under the control of Ole18 and Ltp2 promoter, N= negative control).
Among the various transgenic lines obtained from T1 generation, twenty independent transgenic lines were selected on the basis of genomic PCR analysis and available Pi content (Fig. 57). The transgenic lines exhibiting higher Pi levels were selected and subsequent generations were grown in green house until maturity. On successive screening of the consequent generations, the progenies of IO6-97, IO6-163 derived from Ole18-IPK1 downregulated lines and progenies of IL6-115, IL6-287 derived from Lip2-IPK1 downregulated lines showing higher Pi levels were selected. The selected transgenic lines were screened further to identify the low phytate transgenic line.

![Figure 57: Available Pi content analysis of T1 transgenic lines expressing downregulated IPK1 gene in seeds.](image)

**4.4.5.2 Expression analysis of transgenics**

Semi-quantitative RT-PCR analysis of both transgenic rice (T3 and T4) and non-transgenic control clearly indicated a down-regulation in the transcripts of IPK1 gene as compared to the non-transgenic control (Fig. 58 & 59). However, all the seeds showed same level of expression for the house keeping gene, β-tubulin. For further quantification quantitative real time PCR analysis was performed using SYBR Green (Fermentas). The normalized fold reduction in the levels of IPK1 transcripts varied widely among different progenies of IO6-97, IO6-163, IL6-115 and IL6-287 (Fig. 60). However, maximum reduction of 3.85 fold and 3.66 fold was observed in subline IO6-97-9-4-5 and IL6-287-4-9. The observation reveals a distinct down-regulation of IPK1 suggesting successful silencing mediated by RNAi vectors.
Figure 58: Semi quantitative RT-PCR analysis of T₄ transgenic seeds expressing *Ole18-IPK1-006* vector constructs (IO6 lines). IPK1 transcripts as amplified from seeds of selected transgenic lines (a) IO6-163-10-5 and (b) IO6-97-9-4. (NT= Non-transgenic control, 1= 163-10-5-3, 2= 163-10-5-4, 3= 163-10-5-5, 4= 163-10-5-6, 5= 163-10-5-8, 6= 163-10-5-10, 8= 97-9-4-1, 9= 97-9-4-2, 10= 97-9-4-3, 11= 97-9-4-4, 12= 97-9-4-5, 13= 97-9-4-6).

Figure 59: Semi quantitative RT-PCR analysis of T₃ transgenic seeds expressing *Ltp2-IPK1-006* vector constructs (IL6 lines). IPK1 transcripts as amplified from seeds of selected transgenic lines (a) IL6-287-4 and (b) IL6-115-5. (NT= Non-transgenic control, 14= 287-4-3, 15= 287-4-4, 16= 287-4-6, 17= 287-4-7, 18= 287-4-8, 19= 287-4-9, 20= 287-4-12, 21= 115-5-1, 22= 115-5-2, 23= 115-5-3, 24= 115-5-4, 25= 115-5-6 and 26= 115-5-7).
Chapter 4 RNAi mediated silencing of IPK1 gene to reduce phytic acid

Figure 60: Quantitative real time PCR analysis of T3 and T4 seeds of selected transgenics showing downregulation of IPK1 gene. (a) IO6-163-10-5, (b) IO6-97-9-4, (c) IL6-287-4 and (d) IL6-115-5. The normalized fold expression clearly indicates varied level of silencing in the progenies, the maximum being 3.85 fold as observed in 97-9-4-5.

4.4.5.3 Southern hybridization analysis

Southern blot analysis was done with the identified positive plants of T2 and T3 generation rice of line IO6-97 and IL6-287. The results revealed stable integration of the transgene cassette into the progenies (Fig. 61). The Southern hybridization pattern was same for all the
transgenic plants from progenies of the same transgenic line. However, the non-transgenic control plants did not show any hybridization.

Figure 61: Southern hybridization analysis of \( T_4 \) progenies of IO6-97-9-4 and \( T_3 \) progenies of IL6-287-4. (a) Progenies of IO6-97-9-4 showing hybridization for \( RGA2 \) intron indicating integration of the transgene cassette. (b) Progenies of IL6-287-4 showing integration of \( RGA2 \) intron. However no bands were detected in non-transgenic control (NT). (H= HindIII, E= EcoRI, 1= 287-4-3, 2= 287-4-6, 3= 287-4-9 and 4= 287-4-12)

4.4.6 Biochemical analysis of transgenic rice

4.4.6.1 Phosphorus analysis in seeds

We examined the total phosphorus and available Pi levels in seeds of non-transgenic rice and that of transgenic line IO6-97, IO6-163 and IL6-287. The average total phosphorus content of \( T_4 \) transgenic and non-transgenic seeds was found to be 3.928 mg g\(^{-1}\) and 4.162 mg g\(^{-1}\) respectively. No significant difference was observed between the different transgenic sublines and the non-transgenic control seeds (P>0.05). To further determine the storage form of phosphorus in the seeds, the available Pi levels in non-transgenic and transgenic
seeds were analyzed. The Pi concentration of non-transgenic seeds was 0.179 mg g\(^{-1}\) and constituted 4.33% of the seeds total phosphorus. However, the Pi content of the transgenic seeds of IO6-97-9-4-5, IO6-163-10-5-5 and IL6 287-4-9 was found to be 2.201 mg g\(^{-1}\), 1.791 mg g\(^{-1}\) and 2.046 mg g\(^{-1}\) that constitutes approximately more than 50% of the seed total phosphorus, which is significantly higher than that of the non-transgenic seeds (Fig. 62). Although the seeds exhibited higher Pi levels, they displayed a normal phenotype and no aberrations were observed in their embryo structure.

4.4.6.2 HPLC analysis of seed phytic acid content

To quantify phytic acid levels in the seed extracts of transgenic and non-transgenic control HPLC analysis was performed. The determination of phytic acid was based on replacement of phytic acid with thiocyanate ligand from iron(III)–thiocyanate complex. The chromatogram obtained from HPLC/UV-vis method evidently suggests that the transgenics (showing larger peaks of iron(III)–thiocyanate complex) had lower phytate levels as compared to the respective non-transgenic control, which exhibited smaller peak, signifying higher concentration of phytic acid in seeds (Fig. 63a, 63b & 63c). Mean phytic acid values as calculated from the corresponding peak area was 10.28 mg g\(^{-1}\) for the non-transgenic...
seeds, and 3.16 mg g\(^{-1}\), 5.23 mg g\(^{-1}\) and 4.08 mg g\(^{-1}\) for the transgenic line IO6-97-9-4-5, IO6-163-10-5-5 and IL6-287-4-9 (Fig. 63d). This represented a maximum average reduction in seed phytic acid content of 69% for line IO6-97-9-4-5.

![HPLC traces showing peak of iron III-thiocyanate complex](image)

Figure 63: HPLC analysis of phytic acid content in T\(_4\) transgenic seeds expressing pOle18-IPK1-006 and T\(_3\) transgenic seeds expressing pLtp2-IPK1-006 as compared to non-transgenic control. (a, b, c) HPLC traces showing peak of iron III-thiocyanate complex of (a) non-transgenic and (b and c) transgenic seed after extraction of phytic acid and subsequent reaction with iron III-thiocyanate. (d) Amount of phytic acid in non-transgenic (NT) as compared to T\(_3\) and T\(_4\) low phytic acid transgenic seeds. The symbols * indicates significant differences at P=0.05.

### 4.4.6.3 Seed myo-inositol content

It is already established that phytic acid biosynthesis is closely related to myo-inositol biosynthesis (Hegeman et al., 2001, Panzeri et al., 2011). Hence, in our study we also
examined the effect of silencing the last step of phytate biosynthesis on the seed myo-inositol levels. The GC/MS analyses clearly suggest that there is no significant difference between the myo-inositol content of transgenic (*Ole18-IPK1*-006 and *Ltp2-IPK1*-006) and non-transgenic control seeds (Fig. 64). It therefore proves that downregulation of gene expression of *IPK1* does not have any adverse effect on the seed myo-inositol level which is very important for its positive role in different plant metabolisms and other developmental processes.

![Graph showing myo-inositol content](image.png)

**Figure 64: Myo-inositol content in *IPK1* downregulated low phytate transgenic seeds.** The myo-inositol content of transgenic seeds did not show any significant differences as compared to non-transgenic control (NT). (97-9-4-5= progenies of *Ole18-IPK1* line and 287-4-9= progenies of *Ltp2-IPK1* line).

### 4.4.6.4 Amino acid analysis

In order to assess the effect of silencing *IPK1* on different seed storage proteins of rice, we quantified the individual amino acids by HPLC analysis following AccQ-tag method. The results showed no significant difference between the amount of individual amino acids analyzed from the seeds of T4 transgenics and non-transgenic control (Fig. 65). Therefore it
can be suggested that the seed specific suppression of *IPK1* has not led to any deleterious effects on other seed storage proteins (Fig. 66).

![HPLC chromatograms](image)

**Figure 65:** Amino acid analysis in mature grains of non-transgenic and low phytate transgenic plants. Typical HPLC chromatogram representing peaks of different amino acids (a) Non-transgenic control, (b) IO6-97-9-4-5 and (c) IL6-287-4-9 (1= Asp, 2= Ser, 3= Glu, 4= Gly, 5= His, 6= Arg, 7= Thr, 8= Ala, 9= Pro, 10= Cys, 11= Tyr, 12= Val, 13= Met, 14= Lys, 15= Ile, 16= Leu and 17= Phe).

### 4.4.6.5 Quantification of metal content in seeds

Phytic acid is known to be a potent chelator of divalent cations and therefore renders these metal cations unavailable. Hence we examined the content of different metals in the milled seeds of low phytate T4 transgenic seeds as compared to that of non-transgenic control by
Atomic Absorption Spectroscopy (AAS, Perkin Elmer). The amount of different metal cations was found to be higher in low phytate rice seeds when compared to their respective non-transgenic control (Table 4). Among the different metals analyzed, amount of iron increased, the maximum being observed 12.61 μg g⁻¹ in transgenic seeds of IO6-97-9-4-5 whereas non-transgenic control seeds contain 7.027 μg g⁻¹ (Fig. 67). The results indicate a 1.8 fold increase in the levels of iron in milled seeds of low phytate transgenic seeds (IO6-97-9-4-5).

**Table 4:** Metal content as analyzed from T₄ seeds of *IPK1* downregulated transgenic rice by Atomic Absorption Spectroscopy

<table>
<thead>
<tr>
<th>Metals</th>
<th>Non-transgenic</th>
<th>IO6-97-9-4-5</th>
<th>IL6-287-4-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (μg g⁻¹)</td>
<td>5.321 ± 0.067</td>
<td>7.525 ± 0.087</td>
<td>6.432 ± 0.072</td>
</tr>
<tr>
<td>Iron (μg g⁻¹)</td>
<td>7.027 ± 0.077</td>
<td>12.61 ± 0.223</td>
<td>11.71 ± 0.155</td>
</tr>
<tr>
<td>Zinc (μg g⁻¹)</td>
<td>22.30 ± 0.374</td>
<td>26.62 ± 0.294</td>
<td>26.26 ± 0.231</td>
</tr>
<tr>
<td>Magnesium (mg g⁻¹)</td>
<td>0.574 ± 0.010</td>
<td>0.7325 ± 0.002</td>
<td>0.624 ± 0.006</td>
</tr>
</tbody>
</table>
4.4.7 Morphological analysis of transgenics

4.4.7.1 Seed germination analysis

Reduction of phytate levels is often correlated with pleiotropic effects which frequently affect seed development and germination. Hence, embryo structure was studied in both transgenic and non-transgenic seeds. No abnormality was observed at any stage of development in transgenic seeds with low phytate levels. Furthermore, the seed viability of transgenic seeds as compared to non-transgenic control was assessed by tetrazolium test (Fig. 68). We also examined the seeds germination potential by performing both control germination test and accelerated ageing tests. Germination rate of both transgenics and non-transgenic control seeds were recorded at regular intervals (Table 5). The morphological analysis of seed germination revealed similar phenotype in CGT and AAT of transgenic and control seeds. In addition to this, the activities of important starch degrading enzymes involved in seed germination during optimum conditions were also analyzed. Both transgenic and non-transgenic seeds exhibited similar activities of α-amylase, β-amylase, α-glucosidase enzymes and reducing sugar content (Fig. 69), giving a clear indication that

Figure 67: Iron concentration analyzed from low phytate seeds by Atomic Absorption Spectroscopy. The symbol *** indicates significant differences at P=0.001 with respect to non-transgenic control (NT).
down regulation of phytic acid have not interfered with the seed germination of transgenic rice.

Figure 68: Seed halves showing embryos stained with tetrazolium to check seed viability.

Table 5: Percentage of germination as assessed by CGT and AAT in IPK1 downregulated transgenic seeds as compared to non-transgenic control.

<table>
<thead>
<tr>
<th>Germination (%)</th>
<th>Non-Transgenic</th>
<th>Transgenic- IPK1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGT</td>
<td>98.67 ± 1.333</td>
<td>100 ± 0.000</td>
</tr>
<tr>
<td>AAT- 48h</td>
<td>89.33 ± 1.333</td>
<td>89.33 ± 1.333</td>
</tr>
<tr>
<td>AAT- 96h</td>
<td>40.00 ± 2.309</td>
<td>42.67 ± 1.333</td>
</tr>
</tbody>
</table>

4.4.7.2 Morphological traits of transgenic plants

The agronomic performance of T4 transgenic plants was compared with that of non-transgenic plants (Table 6). Different morphological traits were considered and studied to analyze phenotypic alterations if any in the transgenics. At juvenile stage all T4 and non-transgenic plants showed similar morphologies. In mature plants also no significant difference was observed in plant height, number of tillers and panicles between T4 and non-
transgenic plants ($P \geq 0.05$). The weight of 1000 dry seeds of the transgenics was not significantly different from that of non-transgenic seeds ($P \geq 0.05$). All other morphological parameters considered were similar in both T4 transgenic and non-transgenic plants (Fig. 70).

Figure 69: Biochemical analysis of enzymes involved during germination as analyzed in transgenic low phytate seeds ($IPK1$ down regulated) as compared to non-transgenic control. (a) $\alpha$-amylase. (b) $\beta$-amylase. (c) $\alpha$-glucosidase. (d) Reducing sugar. The transgenic-$MIPS$ represents the mean value as calculated from the observations of seeds of both 106-97-9-4-5 and IL6-287-4-9.
Table 6: Agronomic performance of *IPK1* downregulated transgenics as compared to non-transgenic control

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-transgenic control</th>
<th>IO6-97-9-4</th>
<th>IL6-287-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Height (cm)</td>
<td>126.0 ± 2.082 124.7 ± 2.028</td>
<td>127.0 ± 1.328</td>
<td></td>
</tr>
<tr>
<td>No. of Tillers</td>
<td>12.67 ± 0.333 11.67 ± 0.666 12.37 ± 0.666</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of Effective Tillers</td>
<td>11.33 ± 1.202 11.00 ± 0.577 11.17 ± 0.851</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panicle Length (cm)</td>
<td>26.00 ± 0.577 24.67 ± 0.881 25.33 ± 0.333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grains/Panicle</td>
<td>73.33 ± 1.764 74.67 ± 0.881 73.43 ± 1.186</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry weight (gm)</td>
<td>82.00 ± 0.577 78.33 ± 0.666 81.67 ± 0.481</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed length (mm)</td>
<td>11.67 ± 0.333 11.50 ± 0.288 11.37 ± 0.333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed breadth (mm)</td>
<td>2.613 ± 0.012 2.607 ± 0.014 2.610 ± 0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed length/breadth ratio</td>
<td>4.463 ± 0.142 4.407 ± 0.097 4.357 ± 0.118</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 seeds dry wt (gm)</td>
<td>26.67 ± 0.881 25.67 ± 1.202 26.00 ± 0.577</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.4.8 Introgression of low phytate traits

Twenty hybridized seeds were obtained from hybridization between 97-9-4-5 (*pOle18-IPK1*-006) (male donor) and Swarna (female recipient). The seeds were then germinated and the F₁ plants grown in greenhouse were screened with *RGA2* specific primers to detect the presence of the transgene (Fig. 71). The positive F₁ plants selected were then backcrossed with parental Swarna and the BC₁F₁ seeds obtained have been germinated further and now growing in the green house.
Figure 70: Similar phenotype displayed by transgenic plants expressing IPK1 (106-97-9-4-5) down regulation as compared to non-transgenic control.

Figure 71: Molecular analysis of hybridized plants (IPK1). (M= 1Kb gene ruler; N= Swarna; P= Positive control and Lane 1-9= hybridized plants).

4.5 DISCUSSION

In the present investigation we demonstrated efficient down-regulation of phytic acid, mediated by silencing of IPK1 that catalyzes the last step of the phytate biosynthesis. The enzyme inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IPK1) has been well characterized from different plants viz. Arabidopsis (Sweetman et al., 2006) and maize (Sun et al., 2007).
Furthermore, in *Arabidopsis*, a T-DNA disruption of the InsP$_3$ 2-kinase has been reported to have resulted in an 83% decrease in phytic acid levels (Stevenson-Paulik et al., 2005). Recently Suzuki et al., (2007) elucidated different enzymes involved in phytic acid biosynthetic pathway, among which they reported *IPK1* rice homolog (AK102842) that had 67% similarity to *AtIPK1*. Hence we generated low phytate transgenic rice by silencing the gene expression of *IPK1* by an RNAi mediated approach.

It is already established that phytate globoids occur mainly in the aleurone layer of cereals (Bohn et al., 2008; Regvar et al., 2011). Suppression of enzymes involved in phytate biosynthesis constitutively could be detrimental for plant growth and development. Therefore we used *Ole18* promoter (Qu and Takaiwa, 2004; Kuwano et al., 2009) which has specific activity in the aleurone layer and embryo to drive the suppression of *IPK1* in rice seeds. The transgenics developed showed stable integration of the transgene cassette and displayed a normal phenotype. The expression analysis of transgenic seeds revealed a 3.85 fold reduction in the expression of *IPK1* in the transgenic line IO6-97-9-4-5 with respect to control, suggesting efficient silencing of the gene. In T$_3$ and T$_4$ generation seeds of IO6-97, IO6-163 and IL6-287, the level of available Pi constituted more than 50% of seed total phosphorus which was significantly higher than that of the non-transgenic seeds and phytic acid content was reduced by about 49-69% in different transgenic lines with respect to non-transgenic control. This gives a clear indication that suppression of *IPK1* has actually led to substantial reduction in phytic acid level with a concomitant increase in the amount of available Pi.

In view of earlier reports it is quite evident that low phytate trait is often associated with undesirable effects on embryo structure, seed development and germination potential (Nunes et al., 2006; Kuwano et al., 2009; Doria et al., 2009). Therefore we analyzed the embryo structure of low phytate transgenic seeds during developmental stages and observed no aberrations or anomalies with respect to non-transgenic seeds. Moreover, the assessment of seed germination potential in optimum conditions during control germination tests (Campion et al., 2009) also suggest that the transgenic seeds are viable and showed normal germination pattern with respect to non-transgenic seeds. It was also noteworthy that even during accelerated ageing test (Campion et al., 2009) where seeds of both transgenic and
non-transgenic control were given artificial ageing treatment, the germination potential decreased similarly. The analysis gives a clear idea that though there is reduction in the phytate levels, it has not interfered with either seed development or subsequent germination in both optimum and stressed conditions. To further verify that the germination process was not impaired, we measured the activity of different starch degrading enzymes viz. α-amylase, β-amylase and α-glucosidase, which are also known to be indicator for assessing germination potential in cereals (Galani et al., 2011). All these enzymes showed similar activities in transgenics when compared with non-transgenic seeds indicating normal germination behavior.

The low phytate transgenic seeds were also analyzed for seed myo-inositol content which is considered to be an important metabolite involved in different biochemical pathways that are further associated with important metabolisms in plants (Majumder and Biswas 2006; Torabinejad et al., 2009). In contrast to earlier reports where cereal seeds with low phytate showed lower myo-inositol levels (Panzeri et al., 2011), the transgenic seeds of I06-97 and II6-287 exhibited similar myo-inositol content as compared to non-transgenic seeds. Since IPK1 catalyzes the final step of phytic acid biosynthesis (Suzuki et al., 2007) it is quite obvious that it would not affect the myo-inositol synthesis which occurs much earlier in the pathway. Therefore, this seems to be an added advantage for generating low phytate crops by manipulating IPK1 where myo-inositol levels will not be disturbed. In addition to seed myo-inositol which is an essential metabolite recognized to play significant roles in different signaling pathways, plant growth and development (Abid et al., 2009; Torabinejad et al., 2009), we also evaluated the individual amino acid content of transgenic seeds, since cereal seeds are important source of different seed storage proteins. Hence in order to confirm that seed specific manipulation of IPK1 has not interfered with any of the storage proteins, we analyzed the content of essential amino acids. The results clearly indicated that the amino acid profiles of transgenics were similar to that of non-transgenic control and no significant differences were noticed.

It is already known that phytic acid due to the presence of six highly negatively charged ions chelates the available divalent mineral cations and hence these minerals are less bioavailable (Raboy, 2001; Kumar et al., 2010). Prior reports have suggested that phytic acid chelates
these mineral cations and accumulate them as inclusions in protein storage vacuoles (phytate globoids) mainly in the aleurone layer and embryo (Brinch-Pederson et al., 2007; Raboy 2009). Since, both aleurone layer and embryo are removed during commercial milling lesser amount of these chelated metals are available in the endosperm which is generally consumed (Krishnan et al., 2003; Bajaj and Mohanthy 2005). In view of these facts we analyzed the metal concentration (Ca, Fe, Zn, Mg) of low phytate transgenic seeds (milled), which evidently suggests a significant increase in their concentration in transgenics as compared to the non-transgenic seeds. It was also noteworthy, that among the different metals analyzed, maximum increment was observed in the levels of iron (Fe) which was nearly 1.8 fold more in milled seeds of IO6-97-9-4-5 compared to that of non-transgenic control. Though previous reports have suggested an increase in iron content by using a dual approach of expressing soybean ferritin (Goto et al., 1999) and Aspergillus phytase (Lucca et al., 2001; Drakakaki et al., 2005) in cereals, the present investigation highlights that lowering phytate itself can individually lead to elevate iron levels in rice seeds.