Transcriptome analysis at mid-stage development seeds in litchi with contrasting seed size

Ashish Kumar Pathak (ash29pathak@gmail.com)
Charles University: Univerzita Karlova https://orcid.org/0000-0003-2114-396X

Ritika Sharma
Sardar Swaran Singh National Institute of Renewable Energy: Sardar Swaran Singh National Institute of Bio-Energy

Vishal Nath
ICAR National Research Centre on Litchi

Sudhir P Singh
Center of Innovative and Applied Bioprocessing

Rakesh Tuli (rtuli@pu.ac.in)
Panjab University

Research Article

Keywords: Litchi, transcriptome, seed size, embryogenesis, small seed

DOI: https://doi.org/10.21203/rs.3.rs-252811/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Litchi is a sub-tropical fruit crop with contrasting genotypes bearing fruits with variable seed size. Small seed size is a desirable trait in litchi, as it improves consumer preference and facilitates fruit processing. Seed specific transcriptome analysis was performed in two litchi genotypes with contrasting seed size to identify the genes associated with seed development in litchi fruits. The transcriptomic data from seeds at mid-development stages (16 to 28 days after anthesis) were de-novo assembled into 1,39,608 Trinity transcripts. Out of these, 6,325 transcripts expressed differentially between the two contrasting genotypes. Several putative genes for salicylic acid, jasmonic acid and brassinosteroid pathways were down-regulated in the small-seeded litchi. The putative regulators of seed maturation and seed storage were down-regulated in the small-seeded genotype. Embryogenesis, cell expansion, seed size and stress related Trinity transcripts exhibited differential expression. Further studies can lead to identification and characterization of early regulators of seed size in litchi.

Introduction

Litchi (Litchi chinensis Sonn.) is an important fruit crop of Sapindaceae family, widely cultivated in tropical and sub-tropical regions. The taste, aroma, nutritional value and medicinal properties of juicy aril have made it a popular fruit worldwide (Ibrahim and Mohamed 2015; Huang et al. 2016; Septembre-Malaterre et al. 2016; Kilari and Putta 2017). The development of aril in litchi is favored by disruption in its seed development. As a result, the fruits with small or rudimentary seed develop larger flesh in fruit. Litchi fruits with large pulp and small seed are liked by the consumers and fruit processing industries.

In dicots, generally seed development is initiated after double fertilization of the egg cell and the central cell in ovule, which grows into seed. Inside the seed, the fertilized egg cell (diploid) develops into embryo and the central cell into triploid endosperm. During early development stages, proliferation of endosperm happens, followed by consumption of the endosperm by growing embryo and subsequently, latter’s development into cotyledons. The protective covering of ovule (integuments) develops into seed coat. Numerous physiological events and biochemical reactions have been reported to determine seed mass and composition, and finally the size of seed in fruit (Ohto et al. 2005).

Seed development is influenced by two main mechanisms, parthenocarpy, where fertilization fails, and stenospermy, where fertilization occurs but embryo development is disrupted (Varoquaux et al. 2000). Incidences of both parthenocarpy and stenospermy (post-fertilization embryo abortion) have been observed in litchi cultivars. Embryo abortion in litchi happens at different embryo development stages e.g. globular, heart shaped, torpedo and cotyledon stage (Yu-Shen Liang 2012). Hence, litchi plant is an excellent material to understand molecular mechanism of seed development through differential transcriptomics at different developmental stages of seeds. Generally, the embryo aborts at or after globular stage and develops large aril with small seeded-normal size fruit (Yu-Shen Liang 2012).
We have earlier (Pathak et al. 2016) reported early-stage seed specific transcriptional profiling in two litchi genotypes with contrasting seed size. In this report, we take the earlier study forward. Global genomic expression profiles are now compared for mid-stage seeds of the two contrasting litchi genotypes. A number of genes and pathways whose expression is differentially regulated in small-seeded litchi at 16 to 28 days post anthesis were identified. The genome expression profiles at early- and mid- stage seeds were compared.

**Material And Methods**

**Plant material, DNA and RNA isolation**

Plant materials (developing fruits) were collected from ICAR - National Research Centre on Litchi, Mushahari farm, Mushahari, Muzaffarpur, Bihar, India. In this study, Bedana (B) was chosen as the small-seeded litchi genotype and China (C) as the bold seeded genotype. The two genotypes with contrasting seed size are genetically fairly close (Nei genetic distance of 0.46) (Pathak et al. 2014) as compared to the other popular cultivars in India and grouped in the same cluster as the previously reported litchi transcriptome genotypes. Developing fruits at 16, 20, 24 and 28 days after anthesis (DAA) were harvested, dipped in liquid N$_2$, and stored in -80 °C (Pathak et al. 2016). Seeds were excised from the stored fruits and total RNA was isolated, using Spectrum Plant Total RNA kit (Sigma Aldrich St. Louis, MO, USA), following manufacturer's protocol. DNase I (Sigma Aldrich St. Louis, MO, USA) was used to remove DNA. The integrity of total RNA was confirmed by using gel electrophoresis using BioAnalyzer. TrueSeq libraries were generated and sequenced on Illumina HiSeq 1000 (Illumina, San Diego, CA) platform at Centre for Cellular and Molecular Platforms C-CAMP, Bangalore, India. RNAseq datasets were submitted to NCBI for both genotypes at different seed developmental stages with accession number SRP076766.

**De-novo reference assembly and prediction of transcript expression**

De-novo transcriptome assembly was performed using combined reads obtained from transcriptome sequencing of developing seeds (16, 20, 24 and 28 DAA) in the two contrasting genotypes (B & C). Adapter and low quality sequences were removed, employing Trinity pipeline at default parameters, and the high quality reads were used for assembly. Putative functions were assigned to the assembled Trinity transcripts employing WImpiBLAST tool (Sharma and Mantri 2014) to perform BLASTx homology search against several public databases- NCBI NR, Swissprot, protein databases of the selected plants e.g. *Arabidopsis thaliana, Citrus sinensis, Ricinus communis, Populus trichocarpa, Fragaria vesca, Carica papaya* and *Glycine max*.

Relative expression of Trinity transcripts among RNAseq libraries from two litchi genotypes at four seed developmental stages was obtained as TPM (transcripts per million) and FPKM (fragments per kilobase of transcripts per million mapped reads), using RSEM version 1.2.9 at default parameters (Pathak et al. 2016). Expression levels of different Trinity transcripts were compared among the samples by Edger.
Bioconductor, using script run_DE_analysis.pl as default parameter. The differentially regulated transcripts were retrieved at the cutoff- \( \log_2 \text{fold} \geq 2; P\text{-value} \leq 0.001. \)

**Quantitative real-time PCR analysis**

A few representative, differentially expressed transcripts obtained by high throughput sequencing were validated by quantitative real-time PCR. Total RNA was isolated from seeds at 20 and 24 DAA, and cDNA synthesized from total RNA (500 ng) using oligo (dT) primers and M-MLV reverse transcriptase, according to the manufacturer's instructions (Invitrogen, USA) in a 20 µl volume. Transcript levels were analyzed by quantitative real-time PCR using the fast SYBR green master mix (Applied Biosystems, USA) and an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) according to the manufacturers’ instructions. All biological replicates were analyzed in duplicate. Real-time PCR reactions were normalized to the Ct values for litchi Actin (HQ615689). The relative expression levels of the target genes were calculated using the formula \(2^{-\Delta \Delta CT}.\)

**Results**

**Transcriptome in developing seeds of bold- and small-seeded litchi cultivars**

Transcriptome was sequenced from developing seeds of large-(C) and small (B)-seeded litchi genotypes at 16, 20, 24 and 28 DAA using Illumina paired end sequencing. After stringent quality assessment and data filtering, 334,574,104 clean reads were assembled into 1,39,608 Trinity transcripts (Table. S1). These were matched (BLASTx) with ten different publically available databases (\(E\)-value \(\leq 10^{-5}\)). A total of 56,305 transcripts were annotated, out which 36,099 transcripts matched with the protein coding genes. In BLASTx analysis after NCBI-NR database, the litchi transcripts exhibited highest homology with *Fragaria vesca.* (Table.S2). BLASTn analysis (\(E\)-value of \(10^{-5}\)and query coverage \(\geq 60\ %\)) revealed transcription information of a total of 45,612 Trinity transcripts, which have not been reported previously.

**Differential expression analysis**

A total of 6,325 Trinity transcripts were identified as significantly differentially expressed (\(\log_2\text{fold change} \geq 2; P\text{-value} \leq 0.001\)) in the developing seeds of the two contrasting litchi genotypes. Out of these, 866 transcripts were differentially expressed at all the four developmental stages (16, 20, 24 and 28 DAA). The highest deviation in expression pattern was observed between the bold- and small-seeded seeds at 16 DAA in mid-seed developmental stages (Fig. S1). Highest percentage of differentially expressed genes was observed at 0 DAA (Fig S2). **It supports dominant role of maternal factors in seed size regulation of litchi.** The principal component analysis (Fig. S3) of the differentially expressed transcripts revealed 84.34 % variability by two components. The first component explains 44.44% variability among the developing seeds, whereas the second component accounts for 39.90% variability and grouped the genomic profiles of the bold- and small-seeded litchi genotype separately.
On the basis of homology with *Arabidopsis thaliana*, the genes that expressed differentially were identified at each developmental stage. GO enrichment patterns showed higher representation of transcripts related to biological processes like regulation of primary metabolic process, cellular biosynthetic process, cellular metabolic process etc. in developing seeds of the small-seeded cultivar. This suggests that the seeds in small-seeded litchi are metabolically more active than in the bold-seeded varieties. The down-regulated transcripts in small-seeded genotype showed association with the biological processes, such as post-embryonic development, organ development, anatomical structure development, multicellular organismal development, defense response, protein amino acid phosphorylation etc.

**Expression profile of putative hormone related genes**

Putative hormone related transcripts were predicted in litchi on the basis of homology with *Arabidopsis thaliana*. The BLASTx search revealed the expression of a total of 1,357 hormone related transcripts in the developing seeds. A total of 86, 43, 55 and 72 transcripts were differentially expressed (bold-seeded vs small-seeded) in seeds at 16, 20, 24 and 28 DAA, respectively. Majority of the putative genes for brassinosteroid, jasmonic acid and salicylic acid pathways were down regulated in developing seeds of the small-seeded litchi (Fig. 1, 2 & 3). The putative genes involved in brassinosteroid signal transduction, BSK3 and BAK1 (c2787_g1_i1_AT4G00710 & c66146_g1_i2_AT4G33430), were down regulated in the seeds of small-seeded genotype at all the four developmental stages (Fig. 1). Deficiency of BAK1 leads to induction of necrosis upon infection (Kemmerling et al. 2007). At 24 DAA, the putative NAC 100 (c56916_g1_i1_AT5G61430) and EXO (AT4G08950; c49843_g1_i1, c49843_g2_i1& c49843_g3_i1) genes, reported to regulate cell expansion (Schröder et al. 2009; Pei et al. 2013), showed higher expression in seed of small-seeded litchi (Fig. 1). The putative SAG 29 (c51255_g1_i1_AT5G13170), which may induce cell death under stress (Seo et al. 2011) and organic cation/carnitine transporter 3 (OCT-3) (c54181_g1_i1_AT1G16390) a stress induced gene(Küfner and Koch 2008), were up-regulated at 20 and 28 DAA, respectively, in small-seeded litchi (Fig. 1).

The putative jasmonic acid biosynthesis related gene TPC1 (AT4G03560;c63566_g2_i5, c63566_g2_i6, c63566_g2_i7 & c63566_g2_i10), a key regulator of jasmonic acid positive feedback loop (Bonaventure et al. 2007), exhibited lower expression in the seeds at the four developmental stages in small-seeded litchi (Fig. 2). Higher expression of putative WRKY 70 gene (c5001_g1_i1_AT3G56400), repressor of some of the jasmonic and salicylic acid response (Ülker et al. 2007) genes was observed in seeds of small-seeded litchi at 20 and 24 DAA (Fig. 2). In contrast, at 28 DAA the putative transcripts for jasmonic acid pathway related genes, carboxyl methyltransferase (JMT) (AT1G19640; c53969_g1_i1), DAD1 (c70262_g1_i1_AT2G44810) and MYB23 (c64132_g2_i2_AT5G40330) showed higher expression in small-seeded litchi. The salicylic acid related gene, cysteine-rich RLK 26 (AT4G38830; c58745_g2_i1, c58745_g2_i2, c58745_g2_i3 & c54001_g1_i1) was suppressed in small-seeded seeds at all the analyzed developmental stages (Fig. 3). Putative transcripts for dehydration responsive element B1A (DREB 1) (c50501_g1_i1_AT4G25480) and BSMT1 (c51568_g1_i1_AT3G11480) were up-regulated in small-seeded genotype at 24 and 28 DAA, respectively (Fig. 3). Constitutive expression of DREB 1 in *Arabidopsis* is reported to
enhance cold tolerance (Gilmour et al. 2000). Over expression of *Oryza sativa* BSMT1 in *Arabidopsis* results in higher susceptibility of plants to pathogens (Koo et al. 2007).

**Expression profile of putative transcription factors**

Transcription factors (TFs) control various developmental aspects having direct relation with seed specific traits, such as embryo and endosperm development, maturation etc (McElver et al. 2001; Braybrook and Harada 2008). BLASTx search revealed a total of 2,324 putative TFs involved in advanced stages of seed development in litchi. A total of 118, 45, 76 and 120 putative TF transcripts were differentially expressed (bold-seeded vs small-seeded) at 16, 20, 24 and 28 DAA, respectively (Fig. 4).

At 16 DAA most of the putative TFs showed relatively suppressed transcriptional pattern in small-seeded litchi (Fig. 4). The transcripts related to the TF families, WRKY, WOX, NF-YC, MYB, HSF, bHLH and bZIP, exhibited lower expression in small-seeded seeds at 16 and 28 DAA (Fig. 4). The TF families, RAV, SRS, TALE and TCP exhibited enhanced expression at 28 DAA, while TALE and TCP were down-regulated at 16 DAA in small-seeded litchi (Fig.4).

The expression of WRKY transcription factor family putative genes, involved in giving stress tolerance (Chen et al. 2013; Guo and Qin 2016), such as WRKY 71 (AT1G29860; c35398_g1_i2 & c32185_g1_i1), WRKY 75 (c10716_g1_i1_AT5G13080) and WRKY 53 (AT4G23810; c61726_g2_i2 & c61726_g2_i3) were down-regulated in small-seeded genotype at 16 & 28 DAA (Fig.4). The putative NF-YC11 like transcript (AT3G12480; c63020_g1_i1, c63020_g1_i2 & c63020_g1_i7), required for meristem maintenance (Knuesting et al. 2015), was suppressed in developing seeds of small-seeded litchi. Almost no transcript of putative EDF3 of RAV family (c65483_g5_i1_AT3G25730) genes, promote senescence (Chen et al. 2015); was detected in bold-seeded litchi, while these showed substantial expression in the small-seeded cultivar (Fig.4). bHLH transcription factor family is involved in plant development and responses to the environment (Heisler et al. 2001; Groszmann et al. 2011). The putative BEE1 (c42857_g1_i1_AT1G18400), a brassinosteroid signaling component, a positive regulator of shade avoidance syndrome (Cifuentes-Esquivel et al. 2013) and Abnormal shoot 5 (AT1G68810; c58327_g1_i1, c58327_g1_i2, c58327_g1_i3 & c58327_g1_i5), regulator of plant organ morphogenesis (An et al. 2014) showed lower expression in seeds at 16 DAA in small-seeded litchi (Fig.4).

At 20 DAA, expression of transcription factor families possibly involved in seed development, such as AP2, E2F/DP, G2-like, GRF, MYB, NF-YC and M-type was found repressed in seeds of small-seeded litchi (Fig. 4). The TF families C2H2, NAC, Trihelix and NF-YB were up-regulated in seeds of small-seeded litchi (Fig. 4). The TFs, which are involved in regulation of cell proliferation, cell-wall synthesis, and seed lipid content, TOE1 (c54459_g2_i2_AT2G28550) and WRI (c63744_g1_i1_AT3G54320) (Cernac and Benning 2004), were down-regulated in the small-seeded genotype (Fig. 4). Single mutant of toe1 (*Arabidopsis*) is reported to cause early flowering. Mutant of WRI1 is reported to fail seed oil content in Arabidopsis seeds (Ma et al. 2013).
The TFs families DBB, Dof, GATA, NF-YB, NF-YC, TALE, TCP and WOX were down-regulated; while, ARF, C2H2, HSF and E2F/DP were up-regulated in seeds of small-seeded genotype at 24 DAA (Fig. 4). The DBB family TF, BBX21 (c53932_g1_i1_AT1G75540), which interacts with ABI (ABA- insensitive) genes (Xu et al. 2014) and putative KNAT6 (c48636_g2_i1_AT1G23380) gene of TALE family, involved in boundary establishment during embryogenesis (Belles-Boix et al. 2006), was down-regulated in small-seeded genotype at 24 DAA (Fig. 4). Putative transcription factor WOX 9 (c59097_g1_i1_AT2G33880) an important regulator of embryogenesis (Wu et al. 2007), exhibited low expression at 24 DAA. The transcripts for ARF1 (c56198_g1_i2_AT1G59750) express at a higher level in seed of small-seeded litchi at 16, 20 and 24 DAA (Fig. 4). ARF11 (c63766_g5_i3_AT2G46530) and ARF13 (c63766_g5_i6_AT1G34170) exhibited higher transcription in small-seeded seeds at 24 DAA (Fig. 4). ARF15 (c63766_g5_i4_AT1G35520) exhibited higher expression in small-seeded genotype at 20 and 24 DAA. ARF 15 is an IAA8 interacting transcription factor. It is reported to play role in floral organ development by changes in jasmonic acid level (Wang et al. 2013). Putative transcripts for heat shock transcription factor A2 (HSFA2) (AT2G26150; c67011_g1_i1, c67011_g1_i2, c67011_g1_i4, c67011_g1_i5, c67011_g1_i6 & c67011_g1_i7) were highly expressed in small-seeded litchi genotype at 24 DAA (Fig. 4). At 20 & 24 DAA, putative transcript for WRKY 26 (c48915_g1_i1_AT5G07100) and WRKY 70 (c5001_g1_i1_AT3G56400) were up-regulated in small-seeded genotype (Fig. 4). The putative transcripts of WRKY 41 (AT4G11070; c55590_g1_i1 & c55590_g1_i2) and WRKY 51 (c24833_g1_i1_AT5G64810), were up-regulated in small-seeded genotype at 24 DAA (Fig. 4).

The TF families ARF, B3, NF-YA, NF-YB, NF-YC and MIKC were down-regulated; while, SRS, TALE, TCP and trihelix transcription factor families were up-regulated in small-seeded litchi at 28 DAA (Fig. 4). Contrary to this, the putative SRS7 gene (AT1G19790; c53897_g1_i1, c53897_g1_i4, c53897_g1_i5 & c53897_g1_i6) was up-regulated in small seeded litchi at 28 DAA (Fig. 4).

**Embryogenesis and seed maturation related genes in litchi**

The asymmetric cell division in zygote and differentiation of cells results into embryo development. In the litchi seed, transcripts orthologous to the genes essential for embryo development in *Arabidopsis thaliana* were identified. Altered expression of these genes has been reported to develop defective embryos (Meinke et al. 2008) at pre-globular (AT2G32590, AT5G14760 & AT5G13690), globular (AT3G04340, AT5G16715 & AT1G08840), and cotyledon (AT1G21970, AT3G19700, AT4G30580, AT4G33090, AT1G20200, AT3G06350, AT3G43220, AT3G06350 & AT4G02570) stages. These were down-regulated in the seeds of small-seeded litchi (Fig. 5). The aminopeptidase M1 (APM1) (AT4G33090) was down-regulated at all the mid- developmental stages (16 to 28 DAA) in small-seeded litchi genotype. AT1G08840, AT2G32590, AT3G06350, AT3G20070, AT3G43220, AT4G02570, AT5G13690, AT2G32950 and AT5G16715 were down-regulated at both early (0 to 14 DAA) and mid stage (16 to 28 DAA) stages of litchi embryo development (Fig. 5). The results suggest APM1 (AT4G33090) as a potential gene critical to embryo development in litchi and that leads to seed abortion, without affecting fruit size. It is known to cause embryo abortion at cotyledon stage in *Arabidopsis thaliana*.
Embryogenesis is tightly controlled for the development of different cell identities like the formation of outer (protoderm) versus inner layer, vascular tissues and determination of root and shoots domains. The putative PXL2 gene (c80364_g1_i1_AT4G28650), which regulates the ordered cell division in undifferentiated cells (Fisher and Turner 2007), exhibited low level of expression in small-seeded genotype at 16 DAA (Table.S3). Suppressed transcription was recorded for the seed size regulator, CYP78A6 (24DAA) (c59232_g1_i1_AT2G46660) and IKU2 (16 & 28 DAA) (c62197_g2_i1_AT3G19700) (Garcia et al. 2003; Fang et al. 2012), in the early-stages seeds of small-seeded litchi (Table.S3). Expression of CYP78A6 was less in small seeded genotype at 20, 24 and 28 DAA in this study and at all three developmental stages in our previous report (Pathak et al. 2016). We speculate CYP78A6 as a potential target for seed size determination of litchi.

With the onset of seed maturation, late embryogenesis abundant (LEA) protein accumulates in the seed. The putative transcripts encoding LEA family proteins, AWPM-19 (c53004_g1_i1_AT1G04560) and LEA7 (c54299_g1_i1_AT1G52690) were down-regulated in small-seeded genotype at 28 DAA (Table. S3). At the same time, the seed storage proteins of oleosin family (c42136_g1_i1_AT2G25890, AT3G01570; c15909_g1_i1 & c43018_g1_i1) and 2S albumin family (c23101_g1_i1_AT5G38195) were down-regulated in small-seeded genotype at 28 DAA (Table. S3). This is in accordance with down-regulation of key regulators of seed maturation like ABI3 (AT3G24650) and LEC1 (AT1G21970).

The expression behavior of 144 Trinity transcripts in the mid-stage developing seeds was consistent with that in the developing early-stages seeds (Pathak et al. 2016), reported by us earlier. For example, putative transcripts for disease resistance protein (AT5G35450 & AT5G05400), Chitinase A (class III) (AT5G24090) expressed under environmental stress conditions and galactinol synthase 1 (AT2G47180) were up-regulated, while terpene synthase 21 (AT5G23960), K-box transcription factor (AT1G31150), glycolipid transfer protein (AT2G33470), fructose bisphosphate aldolase 2 (AT4G38970), member of MEKK subfamily (AT5G66850), BR-signaling kinase 3 (AT4G00710) and ankyrin repeat family protein (AT1G10340) were down-regulated in small-seeded litchi during different stages examined i.e. from 0 to 28 DAA. Overexpression of Fructose bisphosphate aldolase 2 enhances biomass accumulation (Uematsu et al. 2012). BR-signaling kinase 3 activates brassinosteroid signaling downstream of BRI (Tang et al. 2008).

Validation of representative results by quantitative PCR

To validate differential expression profiles analyzed from the digital data obtained by RNA-seq, real time PCR was performed on 4 representative transcripts selected for different levels of expression at 20 DAA and 24 DAA. The selected transcripts were AT1G31150, AT4G38970, AT1G71140 and AT2G38000. Out of these, AT1G31150 and AT4G38970 were up-regulated in bold-seeded litchi genotype at mid- and early (Pathak et al. 2016) seed developmental stages. AT1G71140 (transmembrane transport) and AT2G38000 (chaperone protein) are the most up-regulated genes between small and bold seeded genotypes. The expression patterns obtained in real time assay were in agreement with those analyzed from RNA-seq
data (Fig. 6). Hence, the RNA-seq data can be used reliably for relative expression analysis of genes in seed development pathway in litchi.

**Discussion**

The litchi fruits containing fleshy biomass of aril with small seeds being more desirable. A few litchi genotypes have been identified that bear fruits with small seed size (Pathak et al. 2014). Development of small seed in litchi is often due to embryo abortion (Yu-Shen Liang 2012) at different developmental stages. The embryo abortion episodes after globular stage (nearby 20 DAA) lead to significant reduction in seed size without compromising aril growth, suggesting mid-stage seeds as a crucial phase of seed and fruit development in litchi (Xiang et al. 2001). Application of maleic hydrazide at 2 weeks after anthesis, affects embryo development and results in fruits with shrunken seeds and 10% more aril production in the fruit (Menzel and Waite 2005). We examined the transcriptome in the mid-stage developing seeds of contrasting genotypes to obtain molecular insight into seed development in litchi (Fig S3).

The transcriptomic profiles in this work (Fig. S1) as well as the earlier report (Pathak et al. 2016) suggest that seeds of small-seeded litchi are metabolically more active than that of the bold-seeded genotypes. The embryogenesis and defense pathways are compromised in small seeded seeds. Maternal tissue in ovule, under the influence of environmental factors is reported to, play crucial role in determining the seed fate (Sun et al. 2004). Under stress, the developing embryo gets reduced supply of nutrients, eventually resulting into retardation of seed development (BARNABÁS et al. 2008; Lemoine et al. 2013). The suppressed expression of genes associated with brassinosteroid, jasmonic acid and salicylic acid pathways in small-seeded litchi seeds is suggestive of a cellular environment with higher vulnerability to stress, in fruits with reduced seed size in litchi (Figs. 1, 2 & 3).

Hormones regulate exo- and endogenous signals in cellular and developmental processes, including seed development (Gray 2004). An imbalance in hormone signaling results into altered seed development (Goldberg et al. 1994; Chaudhury and Berger 2001; Pignocchi et al. 2009). For example, brassinosteroid concentration in seed development influences integument and endosperm development, affecting seed size and shape in *Arabidopsis* (Jiang et al. 2013; Jiang and Lin 2013). It is known to positively regulate biomass accumulation under stressed (Bajguz and Hayat 2009) and unstressed (Bishop and Yokota 2001) conditions. Lower expression of BSK3 (AT4G00710) and BAK1 (AT4G33430) in litchi indicates suppressed brassinosteroid signaling in small-seeded seeds that may induce necrosis. BSK3 interacts with BRI1 and positively regulates brassinosteroid signal transduction (Tang et al. 2008). Mutants of BRI1 are reported to develop small seed phenotype in Arabidopsis, which is rescued by over expression of BSK3. Null mutants of BAK1 are less sensitive to brassinosteroids in Arabidopsis (Li et al. 2002). Higher expression of NAC100 (AT5G61430) which is a negative regulator of cell expansion in rose (Pei et al. 2013), is in agreement with down-regulation of CYP78A6 (c59232_g1_i1_AT2G46660) and IKU2 (c62197_g2_i1_AT3G19700) in small seeded litchi. The higher expression of the positive regulators of cell death SAG 29 (AT5G13170) (Seo et al. 2011) and stress induced OCT 3 (AT1G16390) (Küfner and
Koch 2008), in small-seeded litchi seed could contribute to seed abortion. Down regulation of APM1 (AT4G33090) at mid stage development suggests that aminopeptidase M1 may play an important role in seed development. Lower expression results in the seed abortion after globular stage, resulting in the reduction in seed size without affecting the fruit size. Jasmonic and salicylic acid hormones are involved in various biotic and abiotic stress responses (Rivas-San Vicente and Plasencia 2011; Wasternack et al. 2013). Jasmonate in young tissues activates vegetative storage proteins and pathogen resistance (Creelman and Mullet 1997). Differential transcription of jasmonic acid or salicylic acid metabolism related genes {TPC1 (AT4G03560), WRKY 70 (AT3G56400), JMT (AT1G19640), DAD1 (AT2G44810), MYB23 (AT5G38830), cystein-rich RLK6 (AT4G38830) and DREB1 (AT4G25480)}, suggests compromised defense response in small-seeded litchi. Differential expression of various TFs during seed development between bold- and small- seeded fruits indicates distinctive pathways involved in deciding the seed size in litchi. The role of the TFs such as WRKY, NAC, bZIP, AP2, and MYB in regulating stress responses in plant tissues has been reported (Wang et al. 2016). These exhibited disparate transcription in the seeds of contrasting litchi genotypes. WOX regulates tissue proliferation during Arabidopsis embryonic development(Wu et al. 2007). It was suppressed in small-seeded litchi seed.

The LEA transcripts, storage proteins and important regulators of seed maturation (FUS5, ABI3 & LEC1) were suppressed in small-seeded litchi; suggesting disturbed seed maturation. The basic body plan of a plant is acquired by the developing embryo which reserves food during embryogenesis, followed by seed maturation (Goldberg et al. 1989). Trinity transcripts homologuous to Arabidopsis thaliana genes for seed development were down-regulated in small-seeded litchi genotype (Fig. 5). These are possible targets for embryo arrest at different developmental stages in litchi. Some of the putative litchi genes regulating defense (galactinol synthase 1 & terpene synthase 21), brassinosteroid signaling (BR-signaling kinase 3) and biomass accumulation (fructose bisphosphate aldolase 2) were differentially expressed in the same pattern at all the studied developmental stages in this study and in our earlier report (Pathak et al. 2016). Further analysis will identify the earliest regulatory genes in these networks.

The transcriptional behavior of brassinosteroid related genes in mid-stage developing seed was similar to that at the early developmental stages (Pathak et al. 2016). The down-regulated transcription of auxin transport related genes in early-stage seeds agrees with the higher expression of senescence related transcripts (Ellis et al. 2005) during mid-stages of seed development in small-seeded litchi. In conclusion, the comprehensive analysis of seed specific transcriptome shows dynamic changes that lead to arrest of seed development of litchi fruits. This investigation furthers our understanding of the molecular mechanism of seed development with contrasting seed size in litchi. The seed specific transcriptome repository will serve as a foundation for pursuing functional genomic studies in litchi.

**Declarations**

**Acknowledgments**
This work was supported by Department of Biotechnology (DBT), Govt. of India. AKP acknowledges DBT fellowship. RT acknowledges J C Bose fellowship. Authors acknowledge Indian Council of Agriculture Research (ICAR), Govt. of India for creation and maintenance of litchi germplasm collection.

Authors’ contributions

R.T. proposed and designed the study. A.K.P. performed the experiments, and analyzed data. R.S performed qRT-PCR experimental analysis. S.P.S guided the experiments and participated in sample collection. V.N guided and participated in plant sample collection. A.K.P. wrote the manuscript, R.T. improved presentation. All authors read and approved the manuscript.

Conflict of interests: The authors declare no conflict of interests.

References

An R, Liu X, Wang R, et al (2014) The Over-Expression of Two Transcription Factors, ABS5/bHLH30 and ABS7/MYB101, Leads to Upwardly Curly Leaves. PLoS One 9:e107637

Bajguz A, Hayat S (2009) Effects of brassinosteroids on the plant responses to environmental stresses. Plant Physiol Biochem 47:1–8. https://doi.org/https://doi.org/10.1016/j.plaphy.2008.10.002

BARNABÁS B, JÄGER K, FEHÉR A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ 31:11–38. https://doi.org/https://doi.org/10.1111/j.1365-3040.2007.01727.x

Belles-Boix E, Hamant O, Witiak SM, et al (2006) KNAT6: An Arabidopsis Homeobox Gene Involved in Meristem Activity and Organ Separation. Plant Cell 18:1900–1907. https://doi.org/10.1105/tpc.106.041988

Bishop GJ, Yokota T (2001) Plants Steroid Hormones, Brassinosteroids: Current Highlights of Molecular Aspects on their Synthesis/Metabolism, Transport, Perception and Response. Plant Cell Physiol 42:114–120. https://doi.org/10.1093/pcp/pce018

Bonaventure G, Gfeller A, Proebsting WM, et al (2007) A gain-of-function allele of TPC1 activates oxylipin biogenesis after leaf wounding in Arabidopsis. Plant J 49:889–898. https://doi.org/https://doi.org/10.1111/j.1365-313X.2006.03002.x

Braybrook SA, Harada JJ (2008) LECs go crazy in embryo development. Trends Plant Sci 13:624–630. https://doi.org/https://doi.org/10.1016/j.tplants.2008.09.008

Cernac A, Benning C (2004) WRINKLED1 encodes an AP2/EREB domain protein involved in the control of storage compound biosynthesis in Arabidopsis. Plant J 40:575–585. https://doi.org/https://doi.org/10.1111/j.1365-313X.2004.02235.x
Chaudhury AM, Berger F (2001) Maternal control of seed development. Semin Cell Dev Biol 12:381–386. https://doi.org/https://doi.org/10.1006/scdb.2001.0267

Chen W-H, Li P-F, Chen M-K, et al (2015) FOREVER YOUNG FLOWER Negatively Regulates Ethylene Response DNA-Binding Factors by Activating an Ethylene-Responsive Factor to Control Arabidopsis Floral Organ Senescence and Abscission. Plant Physiol 168:1666 LP – 1683. https://doi.org/10.1104/pp.15.00433

Chen X, Liu J, Lin G, et al (2013) Overexpression of AtWRKY28 and AtWRKY75 in Arabidopsis enhances resistance to oxalic acid and Sclerotinia sclerotiorum. Plant Cell Rep 32:1589–1599. https://doi.org/10.1007/s00299-013-1469-3

Cifuentes-Esquivel N, Bou-Torrent J, Galstyan A, et al (2013) The bHLH proteins BEE and BIM positively modulate the shade avoidance syndrome in Arabidopsis seedlings. Plant J 75:989–1002. https://doi.org/https://doi.org/10.1111/tpj.12264

Creelman RA, Mullet JE (1997) BIOSYNTHESIS AND ACTION OF JASMONATES IN PLANTS. Annu Rev Plant Physiol Plant Mol Biol 48:355–381. https://doi.org/10.1146/annurev.arplant.48.1.355

Ellis CM, Nagpal P, Young JC, et al (2005) AUXIN RESPONSE FACTOR1 and AUXIN RESPONSE FACTOR2 regulate senescence and floral organ abscission in Arabidopsis thaliana. Development 132:4563 LP – 4574. https://doi.org/10.1242/dev.02012

Fang W, Wang Z, Cui R, et al (2012) Maternal control of seed size by EOD3/CYP78A6 in Arabidopsis thaliana. Plant J 70:929–939. https://doi.org/https://doi.org/10.1111/j.1365-313X.2012.04907.x

Fisher K, Turner S (2007) PXY, a Receptor-like Kinase Essential for Maintaining Polarity during Plant Vascular-Tissue Development. Curr Biol 17:1061–1066. https://doi.org/10.1016/j.cub.2007.05.049

Garcia D, Saingery V, Chambrier P, et al (2003) Arabidopsis haiku Mutants Reveal New Controls of Seed Size by Endosperm. Plant Physiol 131:1661 LP – 1670. https://doi.org/10.1104/pp.102.018762

Gilmour SJ, Sebolt AM, Salazar MP, et al (2000) Overexpression of the Arabidopsis CBF3 Transcriptional Activator Mimics Multiple Biochemical Changes Associated with Cold Acclimation. Plant Physiol 124:1854 LP – 1865. https://doi.org/10.1104/pp.124.4.1854

Goldberg RB, Barker SJ, Perez-Grau L (1989) Regulation of gene expression during plant embryogenesis. Cell 56:149–160. https://doi.org/10.1016/0092-8674(89)90888-X

Goldberg RB, de Paiva G, Yadegari R (1994) Plant Embryogenesis: Zygote to Seed. Science (80- ) 266:605 LP – 614. https://doi.org/10.1126/science.266.5185.605

Gray WM (2004) Hormonal regulation of plant growth and development. PLoS Biol 2:E311–E311. https://doi.org/10.1371/journal.pbio.0020311
Groszmann M, Paicu T, Alvarez JP, et al (2011) SPATULA and ALCATRAZ, are partially redundant, functionally diverging bHLH genes required for Arabidopsis gynoecium and fruit development. Plant J 68:816–829. https://doi.org/https://doi.org/10.1111/j.1365-313X.2011.04732.x

Guo D, Qin G (2016) EXB1/WRKY71 transcription factor regulates both shoot branching and responses to abiotic stresses. Plant Signal Behav 11:e1150404. https://doi.org/10.1080/15592324.2016.1150404

Heisler MG, Atkinson A, Bylstra YH, et al (2001) SPATULA, a gene that controls development of carpel margin tissues in Arabidopsis, encodes a bHLH protein. Development 128:1089 LP – 1098

Huang F, Zhang R, Liu Y, et al (2016) Dietary litchi pulp polysaccharides could enhance immunomodulatory and antioxidant effects in mice. Int J Biol Macromol 92:1067–1073. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2016.08.021

Ibrahim SRM, Mohamed GA (2015) Litchi chinensis: medicinal uses, phytochemistry, and pharmacology. J Ethnopharmacol 174:492–513. https://doi.org/https://doi.org/10.1016/j.jep.2015.08.054

Jiang W-B, Huang H-Y, Hu Y-W, et al (2013) Brassinosteroid Regulates Seed Size and Shape in Arabidopsis. Plant Physiol 162:1965 LP – 1977. https://doi.org/10.1104/pp.113.217703

Jiang W-B, Lin W-H (2013) Brassinosteroid functions in Arabidopsis seed development. Plant Signal Behav 8:e25928. https://doi.org/10.4161/psb.25928

Kemmerling B, Schwedt A, Rodriguez P, et al (2007) The BRI1-Associated Kinase 1, BAK1, Has a Brassinolide-Independent Role in Plant Cell-Death Control. Curr Biol 17:1116–1122. https://doi.org/https://doi.org/10.1016/j.cub.2007.05.046

Kilari EK, Putta S (2017) Delayed progression of diabetic cataractogenesis and retinopathy by Litchi chinensis in STZ-induced diabetic rats. Cutan Ocul Toxicol 36:52–59. https://doi.org/10.3109/15569527.2016.1144610

Knuesting J, Riondet C, Maria C, et al (2015) Arabidopsis Glutaredoxin S17 and Its Partner, the Nuclear Factor Y Subunit C11/Negative Cofactor 2α, Contribute to Maintenance of the Shoot Apical Meristem under Long-Day Photoperiod. Plant Physiol 167:1643 LP – 1658. https://doi.org/10.1104/pp.15.00049

Koo YJ, Kim MA, Kim EH, et al (2007) Overexpression of salicylic acid carboxyl methyltransferase reduces salicylic acid-mediated pathogen resistance in Arabidopsis thaliana. Plant Mol Biol 64:1–15. https://doi.org/10.1007/s11103-006-9123-x

Küfner I, Koch W (2008) Stress regulated members of the plant organic cation transporter family are localized to the vacuolar membrane. BMC Res Notes 1:43. https://doi.org/10.1186/1756-0500-1-43

Lemoine R, La Camera S, Atanassova R, et al (2013) Source-to-sink transport of sugar and regulation by environmental factors . Front. Plant Sci. 4:272
Li J, Wen J, Lease KA, et al (2002) BAK1, an Arabidopsis LRR Receptor-like Protein Kinase, Interacts with BRI1 and Modulates Brassinosteroid Signaling. Cell 110:213–222. https://doi.org/10.1016/S0092-8674(02)00812-7

Ma W, Kong Q, Arondel V, et al (2013) WRINKLED1, A Ubiquitous Regulator in Oil Accumulating Tissues from Arabidopsis Embryos to Oil Palm Mesocarp. PLoS One 8:e68887

McElver J, Tzafrir I, Aux G, et al (2001) Insertional Mutagenesis of Genes Required for Seed Development in Arabidopsis thaliana. Genetics 159:1751 LP – 1763

Meinke D, Muralla R, Sweeney C, Dickerman A (2008) Identifying essential genes in Arabidopsis thaliana. Trends Plant Sci 13:483–491. https://doi.org/10.1016/j.tplants.2008.06.003

Menzel C, Waite G (2005) Litchi and longan botany, production, and uses. CABI Pub., Cambridge, MA

Ohto M, Fischer RL, Goldberg RB, et al (2005) Control of seed mass by APETALA2. Proc Natl Acad Sci U S A 102:3123 LP – 3128. https://doi.org/10.1073/pnas.0409858102

Pathak AK, Singh SP, Gupta Y, et al (2016) Transcriptional changes during ovule development in two genotypes of litchi (Litchi chinensis Sonn.) with contrast in seed size. Sci Rep 6:36304. https://doi.org/10.1038/srep36304

Pathak AK, Singh SP, Tuli R (2014) Amplified Fragment Length Polymorphism Fingerprinting to Identify Genetic Relatedness among Lychee Cultivars and Markers Associated with Small-seeded Cultivars. J Am Soc Hortic Sci 139:657–668

Pei H, Ma N, Tian J, et al (2013) An NAC transcription factor controls ethylene-regulated cell expansion in flower petals. Plant Physiol 163:775–791. https://doi.org/10.1104/pp.113.223388

Pignocchi C, Minns GE, Nesi N, et al (2009) ENDOSPERM DEFECTIVE1 Is a Novel Microtubule-Associated Protein Essential for Seed Development in Arabidopsis. Plant Cell 21:90 LP – 105. https://doi.org/10.1105/tpc.108.061812

Rivas-San Vicente M, Plasencia J (2011) Salicylic acid beyond defence: its role in plant growth and development. J Exp Bot 62:3321–3338. https://doi.org/10.1093/jxb/err031

Schröder F, Lisso J, Lange P, Müssig C (2009) The extracellular EXO protein mediates cell expansion in Arabidopsis leaves. BMC Plant Biol 9:20. https://doi.org/10.1186/1471-2229-9-20

Seo PJ, Park J-M, Kang SK, et al (2011) An Arabidopsis senescence-associated protein SAG29 regulates cell viability under high salinity. Planta 233:189–200. https://doi.org/10.1007/s00425-010-1293-8

Septembre-Malaterre A, Stanislas G, Douraguia E, Gonthier M-P (2016) Evaluation of nutritional and antioxidant properties of the tropical fruits banana, litchi, mango, papaya, passion fruit and pineapple
cultivated in Réunion French Island. Food Chem 212:225–233. https://doi.org/https://doi.org/10.1016/j.foodchem.2016.05.147

Sharma P, Mantri SS (2014) WImpiBLAST: Web Interface for mpiBLAST to Help Biologists Perform Large-Scale Annotation Using High Performance Computing. PLoS One 9:e101144

Sun K, Hunt K, Hauser BA (2004) Ovule abortion in Arabidopsis triggered by stress. Plant Physiol 135:2358–2367. https://doi.org/10.1104/pp.104.043091

Tang W, Kim T-W, Oses-Prieto JA, et al (2008) BSKs Mediate Signal Transduction from the Receptor Kinase BRI1 in Arabidopsis. Science (80- ) 321:557 LP – 560. https://doi.org/10.1126/science.1156973

Uematsu K, Suzuki N, Iwamae T, et al (2012) Increased fructose 1,6-bisphosphate aldolase in plastids enhances growth and photosynthesis of tobacco plants. J Exp Bot 63:3001–3009. https://doi.org/10.1093/jxb/ers004

Ülker B, Shahid Mukhtar M, Somssich IE (2007) The WRKY70 transcription factor of Arabidopsis influences both the plant senescence and defense signaling pathways. Planta 226:125–137. https://doi.org/10.1007/s00425-006-0474-y

Varoquaux F, Blanvillain R, Delseny M, Gallois P (2000) Less is better: new approaches for seedless fruit production. Trends Biotechnol 18:233–242. https://doi.org/10.1016/S0167-7799(00)01448-7

Wang H, Wang H, Shao H, Tang X (2016) Recent Advances in Utilizing Transcription Factors to Improve Plant Abiotic Stress Tolerance by Transgenic Technology. Front Plant Sci 7:67. https://doi.org/10.3389/fpls.2016.00067

Wang J, Yan D-W, Yuan T-T, et al (2013) A gain-of-function mutation in IAA8 alters Arabidopsis floral organ development by change of jasmonic acid level. Plant Mol Biol 82:71–83. https://doi.org/10.1007/s11103-013-0039-y

Wasternack C, Forner S, Strnad M, Hause B (2013) Jasmonates in flower and seed development. Biochimie 95:79–85. https://doi.org/https://doi.org/10.1016/j.biochi.2012.06.005

Wu X, Chory J, Weigel D (2007) Combinations of WOX activities regulate tissue proliferation during Arabidopsis embryonic development. Dev Biol 309:306–316. https://doi.org/10.1016/j.ydbio.2007.07.019

Xiang X, Ou LX, Qiu YP, et al (2001) EMBRYO ABORTION AND POLLEN PARENT EFFECTS IN NUOMICI AND GUIWEI LITCHI. In: Acta Horticulturae. International Society for Horticultural Science (ISHS), Leuven, Belgium, pp 257–260

Xu D, Li J, Gangappa SN, et al (2014) Convergence of Light and ABA Signaling on the ABI5 Promoter. PLOS Genet 10:e1004197
Yu-Shen Liang (2012) Types of aborted seed and quality evaluation of ‘Wuheli’ litchi (Litchi Chinensis Sonn.). African J Agric Research 7:2910–2917. https://doi.org/10.5897/ajar11.1968