Exploring the sound-modulated delay in tomato ripening through expression analysis of coding and non-coding RNAs

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• Background and Aims Sound is omnipresent in nature. Recent evidence supports the notion that naturally occurring and artificially generated sound waves induce inter- and intracellular changes in plants. These changes, in turn, lead to diverse physiological changes, such as enhanced biotic and abiotic stress responses, in both crops and model plants.

• Methods We previously observed delayed ripening in tomato fruits exposed to 1 kHz sound vibrations for 6 h. Here, we evaluated the molecular mechanism underlying this delaying fruit ripening by performing RNA-sequencing analysis of tomato fruits at 6 h, 2 d, 5 d and 7 d after 1 kHz sound vibration treatment.

• Key Results Bioinformatic analysis of differentially expressed genes and non-coding small RNAs revealed that some of these genes are involved in plant hormone and cell wall modification processes. Ethylene and cytokinin biosynthesis and signalling-related genes were downregulated by sound vibration treatment, whereas genes involved in flavonoid, phenylpropanoid and glucan biosynthesis were upregulated. Furthermore, we identified two sound-specific microRNAs and validated the expression of the pre-microRNAs and the mRNAs of their target genes.

• Conclusions Our results indicate that sound vibration helps to delay fruit ripening through the sophisticated regulation of coding and non-coding RNAs and transcription factor genes.

Keywords: Tomato, sound vibration, ripening, ethylene, miRNA, transcriptome.

INTRODUCTION

Intracellular signalling pathways for the appropriate responses are activated in plants upon the reception of stimuli from external factors such as water, light, temperature, wind, microbes and insects (Mishra et al., 2016). The molecular mechanisms that modulate physiological and metabolic processes for the correct response to occur at the right time after the recognition of an exogenous signal are highly complex (Memon and Durakovic, 2014). Of the many external factors, plant responses to physically stimulating factors have rarely been studied, and we only have a basic understanding about these processes, whereas much is known about plant responses to biological and chemical triggers. One such external factor is sound waves, which are measured based on vibration (hertz; Hz) and intensity (decibels; dB) (Dobie and Van Hemel, 2004). Sound waves can be transmitted through the air, gasses, liquids or solid matter (Shipman et al., 2012).

To date, studies investigating communication between living organisms and plant responses to sound, from perception to output, have primarily focused on human, animal and insect behaviour. Few studies have explored the inter- and intracellular responses of plants to sound and the communication between plants and other organisms (Gagliano, 2013; Mishra et al., 2016). There are several obstacles to investigating the effects of sound on plants, including difficulties in (1) utilizing consistent experimental conditions, including stable Hz and dB intensity, (2) determining the proper exposure time in each experiment and (3) removing background noise for the control treatments. We currently do not understand the exact response of plants to sound in nature, and we have only limited information about this process under controlled conditions. The recent development of techniques for optimizing sound wave treatment has made it possible to conduct such experiments. RNA-sequencing (RNA-seq) and further molecular characterization of sound-treated plants have contributed to our understanding of the nature of this plant response.

Sound vibrations promote seed germination and plant growth and have been shown to increase hypocotyl elongation in Oryza sativa (rice) and Cucumis sativus (cucumber) seedlings and in Arabidopsis thaliana (Takahashi et al., 1992; Johnson et al., 1998; Creath and Schwartz, 2004). In addition, the germination rate of Echinacea angustifolia seeds, which were highly dormant, was improved by treatment with 1000 Hz, 100 dB sound waves (Chuanren et al., 2004). Moreover, alcohol
dehydrogenase (ALD) gene promoters were shown to regulate transgene expression in rice seedlings in a frequency-specific manner, and the germination rate, stem growth, fresh weight increase, root system activity and cell membrane permeability in paddy-grown rice increased significantly in response to specific sound frequencies (Bochu et al., 2003; Jeong et al., 2008). The directional growth of the roots of corn seedlings responds to sound due to soil-based vibrations (Gagliano et al., 2012). Sound waves increase the activity of protective enzymes and peroxidase isoenzymes in chrysanthemum (Xiujuan et al., 2003). Moreover, the expression of genes encoding catalase (CAT) and phenylalanine ammonia lyase (PAL) in Coriolum avellana cells increased in response to low-intensity ultrasound (Safari et al., 2013). Sound waves affect the levels of plant hormones such as indole acetic acid (IAA) and abscisic acid (ABA) in chrysanthemum (Bochu et al., 2004). The expression of genes related to plant hormones such as IAA, gibberellic acid (GA), salicylic acid (SA) and jasmonic acid (JA), as well as genes related to various transcription factors and cell signal transduction, was also altered in A. thaliana by 500 Hz sound wave treatment (Ghosh et al., 2016).

In contrast to plant responses to sound waves in growing tissues, the physiological changes in storage organs such as immature fruits exposed to sound waves have only recently been examined. We previously observed delayed ripening in tomato (Solanum lycopersicum) fruits in response to certain sound wave treatments. We also investigated the regulation of ethylene biosynthesis-related genes involved in this delayed fruit ripening process in response to sound wave treatment (Kim et al., 2015, 2016). Treatment with 1 kHz sound waves delays the ripening of tomato fruits by regulating the expression of RIN and HB-1, encoding important transcription factors involved in ethylene biosynthesis (Kim et al., 2015, 2016). Thus, sound waves are thought to regulate plant hormone levels and hormone-related gene expression, thereby affecting the fruit maturation process. However, studies on this topic have been limited to examining the expression of only target or known genes/phenotypic changes during the maturation process, such as from green to red tomato fruit (Grierson et al., 1986; Omboki et al., 2015).

Tomato is a representative model plant system, as well as an economically important fruit crop with excellent human health and nutritional benefits. The entire tomato genome has been sequenced, serving as a rich genomic resource, and both genetic and physical maps and molecular markers are available for tomato, providing a powerful computational pipeline for sequencing and genome annotation. The molecular mechanisms underlying fruit development and the role of ethylene in climacteric fruit ripening are relatively well understood. From a practical viewpoint, further studies aimed at increasing the storage stability of tomato fruit ripening-related genes. We also sought to identify sound-specific biomarkers such as transcriptional mRNAs and non-coding small RNAs such as precursor microRNAs (pre-miRNAs), finding two sound-specific pre-miRNAs that negatively regulate their target mRNAs. Overall, our findings suggest that sound waves modulate inter- and intracellular processes in plants in a sophisticated manner by regulating the expression of mRNAs and pre-miRNAs corresponding to ripening-related target genes, as well as core transcription factor genes.

MATERIALS AND METHODS

Plant materials and treatment conditions

Tomato (Solanum lycopersicum L. ‘Dotaerang’) fruits at the mature green stage (average fruit weight, 172.9 ± 1.7 g) were sampled from a commercial glasshouse facility in Jeongeup and Yong-In, South Korea. Fruits at the mature green stage were visually inspected to ensure that their surface was completely green and were selected according to USDA standards (USDA, 1991). After harvesting, the tomato fruits were transferred to the laboratory and exposed to 1 kHz sound wave treatment for 6 h. The single-frequency signal was generated using Pro Tools M-Powered software (Avid Technology, Burlington, MA, USA). The speaker volume was 80 dB. To block external noise, the experiments were performed in a custom-made soundproof chamber (Korea Scientific Technique Industry Co., Suwon, South Korea). The sound level within the growth chamber was approximately 40 dB, whereas the sound level in a commercial growth chamber generally reaches approximately 80 dB. Tomato fruits were placed in soundproof chambers to prevent the transmission of vibration between samples during sound wave treatment. After the treatment, the fruit samples were transferred to a storage room...
at 23 ± 1 °C and 60 ± 2 % humidity (for an overview of the sound wave treatment procedure, see Fig. 1A). For the control groups, the fruits were exposed to the same conditions except for sound treatments. The fruits were stored for 7 d after 6 h of 1 kHz sound wave treatment and sampled at 6 h, 2 d, 5 d and 7 d after treatment.

Fig. 1. Transcriptomic profiling of sound wave-treated tomato fruit via RNA-seq. (A) Flow chart of steps used to analyse differentially expressed genes in tomato fruit in response to sound wave treatment. (B) Heatmaps illustrating the expression of differentially expressed genes (DEGs) at 6 h, 2 d, 5 d and 7 d after sound wave treatment. (C) GO enrichment analysis of DEGs at 6 h, 2 d, 5 d and 7 d after sound wave treatment. The analysis was performed using DAVID, and the data were visualized using ReviGO software. The threshold of significance was −log (P = 0.05).
Analysis of ethylene production and flesh firmness

Ethylene production were assessed at various time points in 32 tomato fruits per treatment group. Eight tomato fruits were weighed and kept in a sealed 3900-mL plastic container \((n = 4)\) equipped with septa for 1 h. Headspace samples (1 mL) were withdrawn from the container. Levels of ethylene and carbon dioxide were analysed using a gas chromatograph (Varian 450, Varian Inc., Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a thermal conductivity detector (TCD). The level of ethylene was measured using an FID equipped with an active alumina 60–80 mesh column. The injection chamber, oven and detector were maintained at 110, 70 and 250 °C, respectively.

Flesh firmness was determined using a texture analyser (TA plus, Ametek Lloyd Instruments Ltd, Hampshire, UK) equipped with a 5-mm cylindrical probe and a 250-N load cell. The equatorial region of each fruit was cut into 20-mm thick slices, the probe was pressed 10 mm into the mesocarp surface of each slice with a crosshead speed of 2 mm s\(^{-1}\) and the positive peak force \((N)\) was recorded. The flesh firmness of slices derived from 20 fruits per treatment was evaluated at each time point.

RNA-seq experiments

Transcriptome data were generated by performing RNA-seq analysis of 24 tomato samples, i.e. three 1 kHz sound wave-treated samples and three control samples at 6 h, 2 d, 5 d and 7 d. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. The quality and integrity of the RNA were confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under ultraviolet light. The sequencing library was prepared using a TruSeq RNA Sample Preparation kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer’s protocol. In brief, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads, fragmented and converted into cDNA. After the ligation of adapters, the fragments were amplified by PCR. Sequencing was performed on paired end reads \((2 \times 150 \text{ bp})\) using the HiSeq-2000 platform (Illumina).

RNA-seq data processing and computational analysis

To perform reference genome-based analysis, reference genome sequence data for *Solanum lycopersicum* (tomato) were obtained from the National Center for Biotechnology Information (NCBI) genome database (assembly ID: SL2.50). STAR was applied to tissue samples for mapping and to quantify reads to the reference genome (ver. 2.5). The whole data set generated by RNA-seq is available in the NCBI Gene Expression Omnibus public database under the data series accession number GSE106337.

To estimate gene expression levels, counts per million (CPM) mapped reads values were calculated for each sample. The CPM data were normalized using quantile normalization in the R language environment (version 3.2.5). The measured gene expression values were log\(_2\)-transformed and median centred across genes and samples. Gene set enrichment analysis was carried out to identify the most significant gene sets associated with Gene Ontology (GO) biological process (BP) and canonical pathways, in which the significance of over-represented gene sets was estimated by Fisher’s exact test. Gene set enrichment analysis was performed using DAVID Bioinformatics Resources (ver. 6.8), and summarized displays of enriched functions were obtained using Revigo web-based software (Supek et al., 2011). To explore the regulation of genes involved in specific functions, the absolute and average fold differences in their expression were estimated. To estimate absolute fold differences in gene expression, the fold change in expression of a gene between sound-treated and control samples was log\(_2\)-transformed and converted to an absolute value, and then the mean and standard deviation (s.d.) of this value were estimated. The mean and s.d. values were inversely transformed by exponential conversion of \(2 (\text{range of value: 0 ~ +}\infty)\) to produce line plots of expression changes in sound-treated fruits over time. Absolute fold differences indicate the degree of variation in gene expression, regardless of whether it is up- or downregulated. Average fold differences in the expression of genes involved in specific functions were estimated by the same procedure as described for estimation of absolute fold differences, except that log\(_2\)-transformed values were not converted into absolute values. Average fold differences indicate whether gene expression was increased or decreased.

To investigate the target genes of significant miRNAs in tomato, the gene sequences were used as queries against the Tomato Functional Genomic Database (Fei et al., 2011). To explore transcriptional regulators (TRs) that function in tomato, a list of all TRs in the tomato genome was obtained from the iTAK database (Zheng et al., 2016).

RNA extraction and qRT-PCR

Three biological replications were performed per group in quantitative reverse transcription PCR (qRT-PCR) analysis by sampling tomato fruits from individual plants. Immediately after sampling, the fruits were frozen in liquid nitrogen and stored at \(-80 °C\). Frozen fruits were ground into a powder with a pestle and mortar in liquid nitrogen. Total RNA was extracted from 100 mg of frozen ground tissue using a Plant RNeasy Extraction Kit (Qiagen). The total RNA was treated with DNase I (Qiagen). RNA quantification and quality control were performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Approximately 1 \(\mu\)g of total RNA was used for cDNA synthesis using amfiRivert Platinum cDNA Synthesis Master Mix (GenDEPOT, Barker, TX, USA). The qRT-PCR was performed with 2 \(\mu\)L of 5-fold diluted cDNA as a template using AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Daejeon, South Korea) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Relative mRNA and pre-miRNA levels were determined by normalizing the PCR threshold cycle number of each gene with that of the *Ubiquitin3* reference gene. The target genes for qRT-PCR are listed in Supplementary Data Tables S5 and S6. Transcription is shown relative to that at 0 h (expression level = 1), with the *SlyActin* gene serving as an internal control. Each qRT-PCR analysis was repeated three times. Three biological replicates were performed in the experiments. The primers used for
RESULTS

Sound-mediated phenotypic changes in tomato fruit and experimental procedure

To obtain consistent data, the uniformity of the tomato fruit batch used for the experiment needed to be considered. We used a non-destructive method to measure the uniformity before visible fruit changes. We previously measured ethylene production and flesh firmness as an indication of ripening of tomato fruits treated with or without 1 kHz sound waves (Table 1; Kim et al., 2015). Ethylene production was lower in tomato fruits treated with 1 kHz sound waves than in non-treated tomato fruits (Kim et al., 2015). The differences between treatments in ethylene production were most pronounced 12 d after treatment. These results allowed us to maintain the uniformity of tomato fruit ripening affected by sound wave treatment (1 kHz).

In the current study, we investigated the mechanism underlying the effect of sound waves on fruit ripening by analysing changes in genome-wide expression patterns in tomato fruit in response to sound wave treatment. Figure 1A shows an overview of our sound wave treatment procedure. Immature green tomato fruits were treated with 1 kHz sound waves for 6 h, stored at 23 ± 1 °C and observed at 6 h, 2 d, 5 d and 7 d. As previously reported, tomato fruit ripening was delayed in response to sound treatment compared with the untreated control (Fig. 1A).

Transcriptomic characteristics altered by sound wave treatment in tomato fruit

To identify gene expression patterns associated with delayed fruit ripening due to sound wave treatment, we performed transcriptome profiling. We generated RNA-seq data from three sound wave-treated (1 kHz frequency) and three control samples, at specific time points, including 6 h, 2 d, 5 d and 7 d after treatment. We then sought to identify gene sets that were differentially expressed between control and sound wave-treated sample groups at these time points. We identified genes with 1.5-fold or more difference in expression between the treatment and control groups, finding that 5346, 6512, 727 and 2385 genes were significantly differentially expressed at 6 h, 2 d, 5 d and 7 d after sound wave treatment, respectively (Fig. 1B). Of these genes, only two (LOC101254646 [probably encoding flavin-containing monoxygenase 1]) and LOC101260928 (encoding FAD-dependent urate hydroxylase] and three genes [HSBP21 (heat shock protein 21), LOC101245452 (GDSL esterase/lipase A15g33370) and LOC101254847 (proline-rich protein 4-like]) were commonly up- and downregulated, respectively, by sound wave treatment across all time points, whereas many genes showed specific expression patterns at different time points (Fig. S1).

Using four gene lists, including the 5346, 6512, 727 and 2385 genes that were differentially expressed at 6 h, 2 d, 5 d and 7 d after sound wave treatment, respectively, we performed GO enrichment analysis to identify the biological characteristics of these genes using DAVID software. Using the GO BP category in DAVID, we found that many of these genes were involved in functions associated with the cell wall, ethylene, flavonoid, glucon and phenylpropanoid across all sound treatment time points (Fig. 1C). By exploring canonical pathways associated with significant gene sets, we also found that genes involved in zeatin (cytokinin component)-associated functions were significantly enriched at early time points (6 h and 2 d, Fig. S2). These results indicate that differentially expressed genes (DEGs) across time points after sound wave treatment share common biological characteristics, even though few genes were commonly regulated across all time points of sound treatment.

Based on functional enrichment analysis, we measured changes in the expression of genes in response to sound treatment associated with six enriched functional categories: ethylene, zeatin, phenylpropanoid, flavonoid, cell wall and glucan (Fig. 2). When we estimated the absolute fold differences in gene expression between sound-treated and control samples, we found that the variations in the expression of genes involved in ethylene, zeatin, phenylpropanoid and flavonoid were relatively high at early versus late time points and gradually decreased over time (Fig. 2A–D). By contrast, the variations in the expression of genes involved in cell wall and glucan significantly increased in response to sound wave treatment (Fig. 2E, F). We also estimated the average fold differences in expression of these genes, finding that most genes involved in these functions, except ethylene, were downregulated at early time points, followed by a gradual increase over time in response to sound wave treatment (Fig. 2B–F). The average fold differences in expression of the ethylene-associated genes were slightly high at the early time points but gradually decreased over time in response to sound treatment (Fig. 2A).

Validation of DEGs

We chose representative genes from the six biological functional groups identified by RNA-seq analysis and investigated their expression levels and ranges by qRT-PCR (Fig. 3). Like RNA-seq, we performed qRT-PCR analysis to detect the relative expression levels of 1 kHz-treated tomatoes versus the non-treated controls. We classified the DEGs into three major groups: plant hormones, phenylpropanoids and cell wall modification. In the first group, among genes in the ethylene group

Table 1. Measurement of sound wave-elicited ethylene production and flesh firmness as indicators of tomato fruit ripening

<table>
<thead>
<tr>
<th>Sampling time (d)</th>
<th>Ethylene production (ng kg⁻¹ s⁻¹)</th>
<th>Flesh firmness (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 kHz</td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>0.031 ± 0.003</td>
<td>0.040 ± 0.002</td>
</tr>
<tr>
<td>5</td>
<td>0.126 ± 0.060</td>
<td>0.161 ± 0.040</td>
</tr>
<tr>
<td>8</td>
<td>0.515 ± 0.140</td>
<td>0.714 ± 0.140</td>
</tr>
<tr>
<td>12</td>
<td>1.658 ± 0.150</td>
<td>2.906 ± 0.180</td>
</tr>
</tbody>
</table>

Ethylene production and flesh firmness were measured at 1, 5, 8 and 12 d after 1 kHz sound wave treatments. Values are mean ± s.d. (n = 8 for ethylene production experiments and n = 20 for flesh firmness experiments). The experiments were repeated three times.
whose expression level decreased under sound wave treatment as previously observed, the expression of ACS2, ACS4 and ACO4 (of system 2, involved in fruit ripening) also decreased with time after sound wave treatment, validating our experimental conditions, but the expression of ACO4, which is involved in basal level ethylene production in fruit, was not significantly different under sound wave treatment versus the control (Fig. 3A). Similarly, the expression of zeatin-related genes (ZOG-like, IPT1, CKX4 and CYP735A1), which are associated with another hormone, cytokinin, a plant growth hormone important for plant development, decreased at 6 h after sound wave treatment but increased with time and was highest at 7 d (Fig. 3B).

Among the second group of genes, the expression of flavonoid and phenylpropanoid genes related to antioxidation decreased at 6 h after sound wave treatment and gradually increased thereafter. Among these, the expression of the flavonoid-related genes GAME1 and CHI1 and the phenylpropanoid-related gene Laccase decreased significantly after 6 h and increased at 7 d after sound wave treatment compared with the control (Fig. 3C, D). However, the expression levels of the other genes were similar under sound wave versus control treatment.

The third group of genes included cell wall modification-related genes. The expression patterns of these genes were similar to those determined by RNA-seq, especially CHI3, XTH7, which were highly upregulated at 6 h, 2 d and 7 d, respectively, after sound wave treatment (Fig. 3E). However, the expression of EXPI8 decreased overall under sound wave treatment. Finally, glucan-related genes showed similar expression patterns, with the expression of most genes increasing over time after sound wave treatment (Fig. 3F). In particular, the expression of UDP1-like and cel5 increased at 7 d compared with that of the other genes. Taken together, the overall biological functions affected by sound wave treatment were identified
by RNA-seq and verified by qRT-PCR; however, the underlying detailed mechanisms remained to be elucidated.

Exploring transcriptional regulators that are responsive to sound wave treatment

Based on the overall transcriptomic changes induced by sound, we sought to identify core TRs that are in turn regulated by sound treatment. Among the 2608 known TRs encoded in the tomato genome (Zheng et al., 2016), the expression levels of 673 TRs were significantly altered by sound wave treatment. We detected specific expression patterns for 158, 197, 15 and 50 TRs at the 6 h, 2 d, 5 d and 7 d time points, respectively (Fig. 4A). Among enriched canonical pathways involving these TRs, those involved in plant hormone signal transduction were strongly enriched across all sound wave treatment time points (Table S1). Interestingly, among these genes, ethylene-associated genes, such as ETR5, EREB, ERF1, LOC101267105 and ETR6, were often detected across all time points, pointing to the importance of these genes in the response to sound wave treatment. In addition, we carried out gene-to-gene network analysis

Fig. 3. Validation of 24 differentially expressed genes associated with six functional categories in tomato fruit selected based on RNA-seq data after 1 kHz sound wave treatment via qRT-PCR. (A) Ethylene biosynthesis-related genes, (B) zeatin-related genes, (C) phenylpropanoid-related genes, (D) flavonoid-related genes, (E) cell wall-related genes and (F) glucan-related genes. Error bars indicate s.e. of three biological replicates. The values were normalized against the untreated control.
using 673 TRs to construct an interactive gene network of TRs associated with sound treatment. By searching all known interactions among tomato proteins (Fig. S4) from the BioGrid resource (Chatr-Aryamontri et al., 2017), we created an interaction TR network consisting of a number of plant specific teosinte-branched 1/cycloidea/PCF (TCP) TRs (Fig. 4B). In this network, TCP12 specifically functions at 6 h, TCP1, TCP6 and TCP16 at 2 d, and TCP5 and TCP13 at 7 d, whereas TCP20 is not a time-specific TR (Fig. 4B). Among these TRs, TCP12, which is involved in the ethylene signal transduction pathway and is associated with...
tomato fruit ripening (Parapunova et al., 2014), was activated by sound at an early time point (6 h), suggesting that TCP12, along with downstream TRs, functions as a key mediator of delayed tomato fruit ripening in response to sound treatment.

Identification of sound-specific pre-miRNAs and their target genes

In addition to the transcriptomic changes induced by sound, we also explored the associated non-coding RNAs. First, we identified pre-miRNAs associated with sound wave treatment. Among the 172 differentially expressed pre-miRNAs, the expression levels of 12 pre-miRNAs were significantly altered by sound. Among these, five and three pre-miRNAs were specifically differentially expressed at 6 h and 2 d after sound treatment, respectively, and three pre-miRNAs were differentially expressed at both 6 h and 2 d after treatment (Fig. 5A). We also investigated the target genes of these differentially expressed pre-miRNAs by searching a public database containing miRNA-target information in tomato (Fei et al., 2011). Only three pre-miRNAs, MIR6022, MIR6024 and MIR6026, had interactive target genes listed in this database (Table S2).

To validate pre-miRNA expression in our experimental system, we measured the transcriptional expression of precursor MIR6022 (pre-miR6022), MIR6024 (pre-miR6024) and MIR6026 (pre-miR6026). Although pre-miR6026 expression increased at 6 h and 2 d and decreased at 5 and 7 d upon sound wave treatment compared with the control according to our RNA-seq data, only the expression pattern of pre-miR6026 was reproduced at 2 and 5 d in the qRT-PCR experiment (Fig. 5A, B). Interestingly, a pre-miR6026 target gene, Solyc09g018220 (encoding tm-2 protein), showed an opposite expression pattern at 2 and 7 d after sound exposure, indicating a negative correlation between miRNA and target gene expression in response to sound and validating the RNA-seq data (Fig. 5B). Pre-miR6024 showed reduced expression at 6 h upon sound exposure via RNA-seq, and the expression pattern of pre-miR6024 at 6 h was validated by qRT-PCR (Fig. 5B). Its target gene, Solyc10g008240 (encoding putative late blight resistance protein homolog R1B-12%-2C transcript variant X1) showed increased expression at 6 h compared with the control, indicating its negative correlation with pre-miR6024 expression (Fig. 5B). Pre-miR6022 showed increased expression at 6 h and 5 d and reduced expression at 2 d and 7 d in the RNA-seq data (Fig. 5A). Expression of the target gene of miR6022, Solyc01g005780 (encoding receptor-like protein 30), was negatively correlated with pre-miR6022 expression at 2 d, 5 d and 7 d, whereas the expression pattern of pre-miR6022 was consistent with that determined from the RNA-seq data at 2 d, 5 d and 7 d after sound wave treatment (Fig. 5B).

The discrepancy between RNA-seq and qRT-PCR results might have occurred because the harvesting time differed between the RNA-seq samples and qPCR samples. Nevertheless, the expression patterns of pre-miR6022, pre-miR6024 and pre-miR6026 were (partially) negatively correlated with those of their target genes. Collectively, these results suggest that 1 kHz sound vibration treatment regulates the levels of some transcripts via miRNA, whose contribution was shown in response to the sound treatment. This highlighted the need to confirm our findings by analysing additional specific small RNAs.

Landscape of sound-specific tRNAs and other non-coding RNAs

Next, we explored tRNAs with significantly altered expression in response to sound treatment. Of the 809 tRNAs encoded in the tomato genome, the expression levels of 40 tRNAs were significantly altered by sound treatment. Among these, 13 and 13 tRNAs exhibited altered expression specifically at 6 h and 2 d, respectively, while 12 tRNAs exhibited altered expression at both 6 h and 2 d after sound treatment. The two remaining tRNAs exhibited significantly different expression patterns under sound treatment versus the control, although their expression patterns were not time-specific (Fig. S3). All of these tRNAs and the fold differences in their expression levels compared with the control are listed in Table S3.

We also investigated transcriptional changes in long non-coding RNAs (lncRNAs) associated with sound wave treatment. There are 4280 lncRNAs encoded by the tomato genome, among which 868 exhibited significantly altered expression in response to sound wave treatment: 239, 285, 13 and 44 lncRNAs displayed specific expression patterns at 6 h, 2 d, 5 d and 7 d, respectively (Fig. S5). As many lncRNAs in tomato are similar to those in other species, we chose curated lncRNAs whose accession numbers start with ‘NM_’#. We summarized the expression patterns of the curated lncRNAs and found that 17 lncRNAs were significantly associated with sound wave treatment (Fig. 6A; Table S4). Interestingly, a number of signal recognition particle (SRP) RNAs, i.e. the RNA component of the SRP ribonucleoprotein complex that targets proteins to cellular membranes (Rosenblad et al., 2009), along with small nucleolar RNA (snRNA) U3, a member of the SRP RNA family (Riedel et al., 1996), were actively regulated during the early time points (Fig. 6A).

To confirm the relative levels of lncRNA expression found in the RNA-seq data, we conducted qRT-PCR analysis of LOC107546765 (7S RNA for SRP), LOC107546769 (7S RNA), U3 (snRNA U3) and 7SLRNA (SRP 7SL RNA%2C variant B). The expression of all four lncRNAs increased at 6 h and decreased at 2 d in response to sound wave treatment in the RNA-seq data (Fig. 6A). An identical expression pattern was detected by qRT-PCR at the early time points (6 h and 2 d) for LOC107546765 (Fig. 6B). However, the three remaining lncRNAs (i.e. LOC107546769, U3 and 7SLRNA) exhibited heterogeneous changes in expression in response to sound wave treatment not compatible with the RNA-seq data (Fig. 6B), perhaps due to different sample compositions and the lack of samples used to produce the RNA-seq data.

DISCUSSION

Sound is omnipresent throughout the world. Compared with the effects of sound on animals, few studies have focused on the response of plants to sound waves of scientifically optimized strength and vibration. Instead, most experiments in plants have focused on the beneficial and negative effects of sound on plants using different styles of music as the sound source, which included highly complex patterns in terms of frequency and intensity. The plant responses to music (referred to as ‘green music’) were thus not highly reproducible in many cases. However, recent mechanistic studies involving the use of sound waves of a single frequency and certain duration (such as...
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1 kHz for 1 h) combined with cutting-edge multi-omics technology (including transcriptome and proteome analysis) have shed light on how naturally occurring and artificially generated sound waves contribute to phenotypic changes in plants.

In the current study, transcriptome profiling of various samples provided important insights into the mechanism underlying sound-induced delayed ripening in tomato fruit. The application of 1 kHz sound waves was sufficient to modulate the expression
of genes associated with a representative plant senescence hormone, ethylene, as well as cytokinin biosynthesis and signalling pathways and cell wall modification processes. At the initial stage of our project, we also aimed to identify biomarkers for sound-specific delays in tomato fruit ripening. Based on intensive in silico analysis, three miRNAs, MIR6022, MIR6024 and MIR6026, were selected and validated. Collectively, this study broadens our knowledge of the molecular mechanism underlying the delay in tomato fruit ripening induced by a physical trigger, sound waves, and provides the first candidate biomarkers for sound-activated physical changes in plants.

RNA-seq analysis showed that the expression of various genes increased or decreased in response to sound wave treatment, and these genes were classified into six major biological functional groups. Four genes among DEGs in each group were selected and validated by qRT-PCR (Fig. 3). First, the expression of ACS2, ACS4 and ACO4 was reduced by sound wave treatment; these system 2 genes are related to ethylene biosynthesis, which is strongly activated during tomato fruit ripening (Fig. 3A). These results are consistent with the finding that genes related to ethylene biosynthesis are regulated by sound waves and that fruit ripening is delayed by this treatment at the
maturation stage (Kim et al., 2015). Expression of enzymatic genes related to carotenoid degradation was enhanced by sound wave treatment, while expression of genes involved in lycopene biosynthesis was reduced (data not shown). However, these changes were not significant. The genes related to carotenoid degradation include zeaxanthin epoxidase (LOC544162), 9-cis-epoxy-carotenoid-dioxygenase2 (LOC100316877) and carotenoid cleavage dioxygenase 1-like (LOC101251405). The genes related to lycopene biosynthesis include zeta-carotene desaturase (dxs) and 1-deoxy-xyllose 5-phosphate synthase 2 (dzs2) (data not shown). These results clearly indicate that delayed fruit ripening in response to sound wave treatment is not a random process but is instead a sophisticated process controlled by various molecules in fruit. These results will be genetically validated in the future using the ethylene-insensitive npenh tomato mutant.

As shown in Fig. 3B, the expression of zeatin-related genes (a type of cytokinin, an important plant hormone) also decreased at 6 h, followed by an increase over time. Cytokinin is involved in cell division and delays plant senescence in conjunction with ethylene. Specifically, the heterologous expression of the Agrobacterium tumefaciens isopentyl transferase (IPT) gene, encoding an enzyme that catalyses the rate-limiting step of cytokinin production, increased endogenous cytokinin levels in plants (Akiyoshi et al., 1984). In addition, the expression of IPT increases cytokinin production and delays leaf senescence in important crop species such as cassava and cotton (Zhang et al., 2010; Liu et al., 2012).

Intriguingly, the expression of phenylpropanoid and flavonoid-related genes initially decreased and then increased over time in response to sound wave treatment (Fig. 3C, D). Carotenoid and flavonoid genes generated by the phenylpropanoid pathway contribute to fruit pigmentation and disease resistance responses, respectively, in many fruits during the ripening process (Bovy et al., 2007; Singh et al., 2010). In particular, GAME1, a gene associated with flavonoids, is negatively regulated by ethylene during fruit ripening, and its expression predominates in green tissue (Itkin et al., 2011). The delayed transition from green to red fruits might be caused by the orchestrated process of ethylene and flavonoid biosynthesis and signalling.

In addition to altered colour changes in response to sound, sound waves might also play a role in disease resistance by controlling the associated genes. If our hypothesis is correct, sound treatment would alter plant resistance against post-harvest diseases caused by, for instance, Botrytis cinerea and Penicillium spp. Recent evidence indicates that sound waves indirectly affect plant defence mechanisms and directly inhibit the growth of pathogenic fungi, including spore germination and hyphal growth (Jeong et al., 2013). In particular, cell wall-related genes are involved in fruit softening (Brummell et al., 1999; Brummell, 2006). Thus, the increased expression of cell wall-associated genes might affect fruit firmness, delay ripening and offer protection from other external environmental stimuli. Therefore, sound might help plants maintain tension on the cell wall. Together, these findings suggest that sound wave treatment regulates specific gene sets involved in delaying the fruit ripening process to extend fruit freshness and attenuate the senescence process. Although tomatoes were exposed to sound vibrations after harvest in our experiments, it would be interesting to explore whether sound exposure could prolong the vegetative stage in plants or simply attenuate senescence before harvest, which would increase fruit biomass and delay senescence.

Ripening is a developmental process involving the transition from physical maturity to senescence. Senescence is a programmed life cycle in plants: germination gives rise to vegetative maturation to senescence, ultimately leading to death (Hadfield and Bennett, 1997). The fruit ripening process is regulated by a highly sophisticated signalling pathway. Transcriptomic, post-transcriptional, translational and post-translational changes occur during fruit development (Wingerl, 2007). Post-transcriptional changes are regulated by non-coding RNAs such as miRNAs and IncRNAs. Recent studies show that three miRNAs, MIR394, MIR414 and MIR482, are involved in tomato fruit ripening, softening and ethylene responses (Zuo et al., 2012). However, because the miRNAs that are specifically regulated by sound (leading to delayed ripening) are not known, we conducted an analysis to identify these miRNAs, as well as biomarkers that can be used to detect miRNAs whose expression specifically changes in response to sound exposure. In the current study, we did not detect these miRNAs but found novel candidates that are specifically expressed in response to sound. We hypothesize that MIR394, MIR414 and MIR482 enhance the ripening process while other miRNAs suppress this process. Of the candidate miRNAs identified, MIR6022, MIR6024, MIR6026 and MIR6027 were previously shown to be expressed during fruit ripening, and they target signal transduction and immune response-related genes (Karlova et al., 2013). We confirmed that the expression of pre-MIR6022, pre-MIR6024 and pre-MIR6026 was altered in response to sound treatment, and the expression of target genes that function in the immune response was negatively correlated with pre-miRNA expression (Fig. 5).

miR6022 targets genes encoding receptor-like proteins (RLPs) (Soly01g005780, Soly01g005760, Soly01g009690) and U-box domain-containing protein (Soly09g018230) (Table S2). Recent studies show that miR6022 shares the most target genes with miR825, such as RLP genes (Soly01g006550.2, Soly01g005730, Soly01g014160.1, Soly03g082780.1) (Wang et al., 2017). miR6024 targets encoding putative late blight resistance proteins (Soly02g070410, Soly10g008240, Soly05g009740), ABC transporter B family members (Soly09g009910) and CC-NBS-LRR resistance proteins (Soly02g084890, Soly09g02290) were detected in the present study (Table S2). In addition, genes for CC-NBS-LRR resistance protein (Soly11g069020.1, Soly05g008070.2, Soly11g071420.1), NBS resistance protein (Soly11g070000.1) and some uncharacterized proteins (Soly11g006520.1, Soly11g020100.1) were recently found to be targeted by MIR6024 (Wang et al., 2017). We identified two target genes of MIR6026, encoding tm-2 protein (Soly09g18220) and CC-NBS-LRR resistance protein (Soly09g02290) (Table S2); tm-2 functions in Tomato mosaic virus (ToMV) resistance (Weber et al., 1993). Therefore, we chose the RLP gene Soly01g005780, the most frequent target of MIR6022, the late blight resistance protein gene Sly10g008240, which is specifically targeted by MIR6024, and the tm-2 gene Sly-09g018220, a fungal and viral resistance gene that is specifically targeted by MIR6026, for validation.

Of the target genes, the reduced expression of the gene encoding LRR-RLK (LRP) was interesting, as LRP is also involved in plant developmental processes such as the formation of vascular tissue, another development and meristeme maintenance process (Hazak and Hardtke, 2016). Thus, the reduced
expression of the LRP gene might lead to delayed plant development. Therefore, the delayed ripening in tomato exposed to sound might be partially due to miRNA-induced suppression of LRP expression. There is also other evidence linking resistance (R) genes to plant development. The wheat *Lr34* gene, conferring rust or powdery mildew resistance, is expressed only in adult wheat plants (Rinaldo et al., 2017). The expression of R genes can be induced depending on the developmental stage, such as ‘senescence-induced resistance suppression’ (Develey-Riviere and Galiana, 2007). Collectively, it appears that plant defence genes such as R genes can be specifically regulated during developmental processes such as ripening. Plants must consume limited energy resources at the proper times, an important step for survival. Prior to ripening, plants use energy to maintain the green colour of their fruit rather than to express R genes. Therefore, it appears that, in tomatoes exposed to sound vibration, the expression of miRNAs that inhibit the expression of these R genes increases, thus keeping the fruits green for a longer period of time and delaying ripening.

In addition, fruit ripening is related to ethylene production in climacteric fruits (Alexander and Grierson, 2002; Jung et al., 2017). Although ethylene controls the terminal development of fruits, it also controls various defence responses (Díaz et al., 2002; Alexander and Grierson, 2002). Our RNA-seq analysis showed that sound vibration delays the ripening process, reducing the expression of genes such as ethylene- and cell wall-related genes. The delayed tomato fruit ripening may be due to the reduced expression of immune-associated genes, including ethylene genes. Three miRNAs whose expression was altered by sound vibrations appear to delay the ripening process by inhibiting the expression of R genes; these miRNAs could be used as sound vibration-specific biomarkers for inhibited fruit ripening.

In addition to the involvement of miRNAs in sound-mediated delayed ripening, we identified IncRNAs associated with sound treatment to identify SRP RNAs and snRNAs (Fig. 6B). U3 snRNA, which is involved in pre-rRNA processing, is grouped together with SRP in the polymerase III-specific subclass of externally regulated poll III genes (Riedel et al., 1996). The SRP is a ribonucleoprotein complex containing an SRP RNA component, which participates in the targeting of protein to the cellular membrane (Rosenblad et al., 2009), to which the SRP recognizes signal sequences and directs secretory proteins (Andersen et al., 2006), suggesting that SRP RNA might function as a mediator controlling protein secretion at the cell membrane in response to sound wave treatment.

This study has several limitations. First, only 24 tomato samples were analysed by RNA-seq, i.e. three control samples and three sound wave-treated samples at four time points. This was insufficient to conclusively determine the role of genes in the effects of sound treatment on fruit ripening. Second, due to the small number of samples per group, we were unable to apply rigorous statistical tests and instead selected DEGs by calculating fold differences in gene expression, which may have led to false positive results. The main objective of this study was to provide biological insight into the effects of sound wave treatment on fruit ripening. After selecting genes associated with sound treatment by calculating fold differences in gene expression, we performed statistical analyses and explored published bioinformatics resources.

In conclusion, we performed the first investigation of the molecular mode of action during sound-mediated delayed fruit ripening and obtained new insights into this process by identifying plant hormone- and cell wall-related transcriptomic changes in response to sound waves. More interestingly, we found that this process is also mediated by diverse epigenetic changes through the actions of IncRNAs and their target miRNAs/transcription factors. Our results support the notion that sound waves could be used as a physical modulator of post-harvest fruit by fine-tuning the ripening process based on our detailed understanding of the physical responses of plants to sound exposure. The use of sound waves has diverse advantages over chemical modulators for application to crops, such as their long-lasting effects without the need for additional input and their stable effectiveness. Because maturation and ripening are crucial steps for post-harvest agricultural product management, we will further explore the expression of various genes regulated by sound wave treatment in plants and the mechanisms identified in this study, which could play a major role in maintaining and improving the quality of agricultural products.

**SUPPLEMENTARY DATA**

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Fig. 1: Comparisons of differentially expressed genes 6 h, 2 d, 5 d and 7 d after sound treatment using a Venn diagram approach. Fig. 2: Significantly enriched canonical pathways (A) 6 h, (B) 2 d, (C) 5 d and (D) 7 d after sound treatment. Fig. 3: Expression patterns of tRNAs associated with sound wave treatment. Fig. 4: All known gene-to-gene interactions in the tomato genome. Fig. 5: Expression patterns of long non-coding RNAs (IncRNAs) associated with sound wave treatment. Table S1: Significantly enriched canonical pathways (A) 6 h, (B) 2 d, (C) 5 d and (D) 7 d after sound treatment. Table S2: Significant miRNAs and their target genes. Table S3: Significant tRNAs by sound treatment. Table S4: Significant IncRNAs by sound treatment. Table S5: Primer sequences used in this study. Table S6: Primer sequences of miRNA-target genes.

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**LITERATURE CITED**


