Sound waves delay tomato fruit ripening by negatively regulating ethylene biosynthesis and signaling genes

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\textbf{A B S T R A C T}

Regulation of tomato fruit ripening may help extend fruit shelf life and prevent losses due to spoilage. Here, tomato fruit were investigated whether sound treatment could delay their ripening. Harvested fruit were treated with low-frequency sound waves (1 kHz) for 6 h, and then monitored various characteristics of the fruit over 14 days at 23 ± 1°C. Seven days after the treatment, 85% of the treated fruit were green, versus fewer than 50% of the non-treated fruit. Most of the tomato fruit had transitioned to the red ripening stage by 14 days after treatment. Ethylene production and respiration rate were lower in the sound-treated than non-treated tomatoes. Furthermore, changes in surface color and flesh firmness were delayed in the treated fruit. To investigate how sound wave treatment effects on fruit ripening, the expression of ethylene-related genes was analyzed by quantitative real-time RT-PCR analysis. The expression level of several ethylene biosynthetic (ACS2, ACS4, ACO1, E4 and E8) and ripening-regulated (RIN, TAGL1, HB-1, NOR, CNR) genes was influenced by sound wave treatment. These results indicated that sound wave treatment delays tomato fruit ripening by altering the expression of important genes in the ethylene biosynthesis and ethylene signaling pathways.© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

\textbf{1. Introduction}

Plants are frequently exposed to abiotic and biotic environmental stresses. Stress signals are transmitted to cells, where they activate various stress response mechanisms. The mechanism by which plants recognize external signals and then alter the expression of genes to affect physiological and/or metabolic processes is complex. Much research has examined the signal transduction mechanisms that regulate the defense or growth responses of plants following artificial stimulation, such as mechanical stimulation (Lin et al., 2009; Braam, 2004; Monshausen and Gilroy, 2008). Other such external signal is sound.

Previous reports on the effects of sound on plants failed to specify the exact experimental conditions (Weinberger and Measures, 1978). Sound vibrations stimulate seed germination and plant growth and increase the rate of hypocotyl elongation in \textit{Oryza sativa} (rice) and \textit{Cucumis sativus} (cucumber) seedlings (Takahashi et al., 1992) and in Arabidopsis (Johnson et al., 1998). Jomdecha and Prateepasen (2011) showed that low-intensity ultrasonic sound enhances the growth of \textit{Saccaromyces cerevisiae}. Furthermore, transgenic rice plants harboring an \textit{Alcohol dehydrogenase (Ald)} promoter:GUS reporter construct showed a significant increase in \textit{GUS} expression after sound treatment at 125 Hz, 250 Hz, or 1 kHz. By contrast, these plants showed a significant decrease in \textit{GUS} mRNA levels after a treatment of 50 Hz, suggesting that the Ald promoter responds to sound in a frequency-specific manner (Jeong et al., 2008). In addition, through biochemical analyses, Bochu et al. (2004) reported that sound wave stimuli (1.4 kHz, 95 dB, 10 days) were associated with increased levels of indole acetic acid (IAA) and decreased levels of abscisic acid (ABA) in \textit{Chrysanthemum}. Accordingly, sound waves are thought to regulate a variety of plant hormones and genes.

Fruits are divided into climacteric and non-climacteric types based on their respiration patterns during ripening. Climacteric fruits, such as tomato, apple, avocado, melon, pear, kiwifruit, and peach, exhibit increased respiration and ethylene production during ripening. However, respiration and ethylene production rates barely change during ripening of non-climacteric fruit, such
as grapes, cherry, strawberry, pineapple, and citrus (Lin et al., 2009; Cara and Giovannoni, 2008). Almost all fruits start aging rapidly when ethylene production increases and once fruit have ripened their quality declines. Suppression of senescence in fruit results in a longer shelf life. Tomato is a major model system for the study of the molecular mechanisms underlying fruit development and the role of ethylene in climacteric fruit ripening. It has various useful characteristics, such as efficient genetic transformation, distinct ripening phenotypes, small genome size, short generation time, and simple diploid genetics (Liu et al., 2014; Giovannoni, 2004).

Ethylene is a plant hormone involved in fruit, flower, and leaf senescence. The ethylene biosynthesis precursor S-adenosylmethionine (SAM) is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and ACC oxidase (ACO) then catalyzes the synthesis of ethylene (Yang and Hoffman, 1984; Johnson and Ecker, 1998). Ethylene biosynthesis in tomato fruit can be divided into systems 1 and 2 during climacteric fruit ripening (Lelièvre et al., 1997; Alexander and Grierson, 2002). System 1 is auto-inhibitory and functions during normal growth and development and the stress response, whereas system 2 operates during floral and leaf senescence and fruit ripening (Katz et al., 2004; Barry and Giovannoni, 2007).

Expression of LeACS2, LeACS4, and LeACO1 increases significantly during fruit ripening, indicating that these genes are major regulators of ripening (Hoogstrate et al., 2014; Lincoln et al., 1993). The ethylene-inducible genes E4 and E8 also have important functions during fruit ripening. Transcription of E4 is rapidly activated by ethylene in both leaves and fruit. By contrast, expression of E8 is highly induced by ethylene in tomato fruit, but is not induced in response to ethylene in leaves. Although E4 and E8 are coordinately expressed during fruit ripening, E8 is controlled both by ethylene and by separate developmental signals, whereas E4 expression is strictly dependent on ethylene (Kesanakurti et al., 2012; Lincoln and Fischer, 1988). Furthermore, TOMATO AGAMOUS-LIKE 1 (TAGL1) MADS box gene as a transcription factor plays an important function in both fruit expansion and ripening. Silencing of TAGL1 does not affect floral organs, but affects ripening of the tomato fruit (Vrebalov et al., 2009; Giménez et al., 2010; Pan et al., 2010). RIPENING-INHIBITOR (RIN) functions upstream of ethylene in the ripening cascade and determine the competence of fruit to ripen. RIN was shown to regulate LeACS2 and LeACS4 expression (Vrebalov et al., 2002). Consequently, TAGL1 and RIN affect ethylene metabolism (Yokotani et al., 2004). In addition, virus-induced gene silencing of LeHB-1, which encodes an HD-Zip homeobox protein, reduced LeACO1 mRNA expression and inhibited ripening in tomato fruit (Lin et al., 2008). In this study, tomato fruits were investigated whether sound wave treatment could delay ripening by regulating the expression of genes involved in ethylene biosynthesis and signaling.

2. Materials and methods

2.1. Plant materials and treatment conditions

Tomato (Solanum lycopersicum L. cv. Dotaerang) fruits were sampled at the mature green stage of development (average fruit weight 172.9 ± 1.7 g) from a commercial greenhouse facility at Yong-in, Korea. After transport to the laboratory of the Rural Development Administration on the day of harvest, the fruits were exposed to a 1 kHz sound wave for 6 h. The single-frequency signal was generated using Pro Tools M-Powered software (Avid Technology, USA). The speaker volume was 100 dB. To prevent extraneous noise, experiments were conducted inside a custom-made, sound-proof chamber (Korea Scientific Technique Industry Co., Korea). The sound level within the chamber was approximately 40 dB, while the sound level in a commercial growth chamber reaches generally about 80 dB. Tomato fruits were placed in the sound-proof chambers to prevent transfer of vibrations between samples during the sound wave treatments. The sound wave treatment was conducted at 23 ± 1 °C. Control groups were achieved with the exposure of fruit in the same conditions except for the sound interference. Fruit samples were placed in a storage room at 23 ± 1 °C and monitored for quality changes after sound wave treatments.

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**Fig. 1.** Comparison of fruit surface color change in response to various sound treatments in tomato. Phenotypic analysis of tomato fruit ripening at 9, 12, 14, and 17 days after a 6-h sound treatment (non-treatment, 250 Hz, 500 Hz, 800 Hz, 1 kHz, and 1.5 kHz). Fruit were maintained at 23°C throughout the experiment. The average results of three experiments are shown.
2.2. Ethylene production and respiration rate determination

Thirty-two tomato fruits per treatment were used for assessing ethylene production and respiration rate at each measurement interval. Eight tomato fruits were weighed and sealed in a 3900 mL plastic container \( (n = 4) \) equipped with a septum for 1 h. Headspace samples (1 mL) were withdrawn from the container and ethylene and carbon dioxide concentrations were analyzed using a gas chromatograph (Varian 450, Varian Inc., CA, USA) equipped with a flame ionization detector (FID) and thermal conductivity detector (TCD).

Ethylene levels were measured by a FID equipped with an active alumina 60–80 mesh column. The injector, oven, and detector were maintained at 110, 70, and 250 \(^\circ\)C, respectively. Respiration rate was determined based on carbon dioxide levels using a TCD equipped with an active carbon 60–80 mesh column. The injector, oven, and detector were maintained at 110, 70, and 150 \(^\circ\)C, respectively. Helium \( (30 \text{ mL min}^{-1}) \) was used as the carrier gas for ethylene and carbon dioxide detection.

Fig. 2. Ripening delay in tomato fruit subjected to a sound treatment of 1 kHz. (A) Phenotypic analysis of tomato fruit ripening at 1, 2, 5, 7, 12, and 14 days \((23 \pm 1 \, ^\circ\text{C})\) after sound treatment \((1 \, \text{kHz})\). (B) The percentage of green tomato fruit during the 14 days \((23 \pm 1 \, ^\circ\text{C})\) following sound treatment \((1 \, \text{kHz})\). Error bars represent SD of three biological replicates.
Tomato fruits were obtained from at least three individuals per step. Directly after harvesting, the fruits were frozen in liquid nitrogen and stored at −80°C. These fruits were ground into powder with pestle and mortar in liquid nitrogen to keep the tissue frozen. Total RNA was extracted using a Plant RNeasy Extraction Kit (Qiagen). Total RNA was treated with DNase I (Qiagen) and cDNA was generated using ampiRiVert Platinum cDNA Synthesis Master Mix (GenDEPOT, South Korea). Quantitative real-time RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara) and the StepOnePlus Real-Time PCR System (Applied Biosystems). The relative mRNA levels were determined by normalizing the PCR threshold cycle number of each gene with that of the Ubiquitin3 reference gene. Three biological replicates were used in the experiments. The primers used for PCR are shown in Table S1.

3. Results and discussion

3.1. Sound wave treatment delayed ripening in tomato fruit

Sound wave significantly affects the ripening process of tomato fruit. To evaluate the impact of sound waves on fruit ripening, tomato fruits at the mature green stage were treated with sound waves of 0 Hz, 250 Hz, 500 Hz, 800 Hz, 1 kHz, and 1.5 kHz for 6 h. For all of the sound wave treatments except for 800 Hz and 1.5 kHz, ripening was delayed as compared with the non-treated control (Fig. 1). Since the 1 kHz sound wave showed the strongest effect on ripening process, tomato fruit were treated with 1 kHz sound for phenotypic analysis over 14 days of storage at 23 ± 1°C. Thus, most of the results in this study were obtained using tomato fruit treated with 1 kHz, unless otherwise indicated. Under these conditions, the mature green stage tomato fruit transitioned to the breaker stage after 2 days. Tomato fruit treated with sound waves of 1 kHz exhibited delayed ripening as compared with the non-treated fruit at 5 or 7 days after treatment (Fig. 2A). Seven days after treatment, over 85% of the treated fruit was still green, whereas less than 50% of the non-treated tomato fruit were green (Fig. 2B). Finally, most of the tomato fruit transitioned to the red ripening stage by 14 days after treatment. These results indicate that sound wave treatment delays tomato fruit ripening.

These findings could be explained partially with the previous reports on the effects of sound wave to the deformability of plant cell. Sound wave exposure changes the deformability of the plant cell membrane, and the deformability is closely related to the frequency of the sound wave (Bochu et al., 2001).

3.2. Sound wave treatment reduced ethylene production and respiration rate in tomato fruit

External stimuli like sound wave also could regulate various biological process and genes. A scientific report by Johnson et al. (1998) reveals that mechanotransduction occurs in cells and tissues, and external mechanical stresses stimulate various signal transduction mechanisms. In this study, external stimuli with the sound wave of 1 kHz applied to 32 tomato fruits at various time points after treatment (1–15 days) prior to the determination of ethylene production. As shown in Fig. 3A, ethylene production was lower in tomatoes treated with sound waves of 1 kHz than in the non-treated control. The ethylene production was observed the greatest reduction at 12 days after treatment. These results indicate that the imposition of the sound treatment wave significantly effects on the biological process of ethylene, resulting in the delay of the fruit ripening. These findings agree well with the previous postharvest physiology of tomato fruit suggested by Lin et al., (2009) and Cara and Giovannini (2008). The ripening of tomato fruit, as a climacteric type fruit, is associated with increased respiration and ethylene production.

Next, the respiration rate was measured in tomato fruit treated or not with sound waves. To examine the respiration rate, the evolution rate of carbon dioxide was analyzed using a gas chromatography. The respiration rate was lower in the treated tomato fruit one day after treatment than in the non-treated control, and the greatest relative reduction was observed at 12 days after treatment (Fig. 3B). In the non-treated fruit, the respiration rate peaked at 12 days, and then decreased rapidly. At this stage,
3.4. Sound wave treatment affects ethylene biosynthesis-related genes

This study found that sound wave controls the expression of genes involved in the biochemical processes associated with ripening. Ethylene mediates certain developmental processes, most notably early fruit development and ripening. Having established that sound wave treatment delayed tomato fruit ripening (Figs. 1–4), tomato fruit ripening assumed to be influenced ethylene-related responses by sound wave treatment. Therefore, the expression pattern of major genes involved in ethylene biosynthesis was analyzed after sound wave treatment. The transcript levels of ethylene biosynthetic genes in sound wave-treated tomato fruit was examined at various time points after treatment (at 23 ± 1 °C) via quantitative real-time RT-PCR analysis with the gene-specific primers listed in Table S1. Under normal conditions, most of the ethylene biosynthetic genes and ripening regulated genes in tomato were expressed at low levels in green fruit, but the transcription levels of each of these genes increased at the onset of ripening, as the fruit started to produce ethylene (Fig. 5). As shown in Fig. 5, in compare to control, the expressions of LeACS2, LeACS4 and LeACO1 (ethylene biosynthesis-related genes) were reduced in sound wave treated tomatoes. The difference of expression level of these genes between treated and untreated groups substantially increased from 7 to 14 days. The expression level of E4 and E8 genes (ethylene-inducible genes) were also significantly reduced by the sound wave treatment. These genes associated with ethylene biosynthesis exhibited the most striking changes in expression between 5 and 7 days after the sound treatment. In particular, the expression of LeACS2, LeACS4, and LeACO1 was markedly reduced by sound treatment, and this reduction in expression is expected to reduce ethylene production. The expression level of E4 and E8 genes (ethylene-inducible genes) were also significantly reduced by the sound wave treatment (Fig. 5), probably due to the low concentrations of ethylene in sound-treated tomato fruit. These results well agree with the previous report on the strict dependence of those target genes with ethylene during fruit ripening (Kesanakurti et al., 2012; Lincoln and Fischer, 1988).

3.5. Sound wave treatment triggers expression of ripening-related genes

RIN, LeHB-1, CNR, and NOR encode transcription factors that regulate the expression of several genes responsible for ethylene biosynthesis. RIN belongs to the MADS-box family of transcription factors, which are important regulators of vegetative growth, flowering time, and floral development (Vrebalo et al., 2002). LeHB-1, a class-I HD-Zip gene, encodes a transcription factor that binds to the promoters of ethylene biosynthesis-related genes that have recently been identified (Lin et al., 2008). In addition, the TOMATO AGAMOUS-LIKE 1 (TAGL1) MADS box gene encodes a

![Fig. 4. Sound treatment causes changes in surface color and hardness in tomato fruit.](image-url)
transcription factor that plays an important function in both fruit expansion and ripening. TAGL1 promotes ripening by activating ACS2, and is reported to be a target of RIN (Itkin et al., 2009; Vrebalov et al., 2009). Silencing of TAGL1 or RIN delays ripening (Vrebalov et al., 2009; Giménez et al., 2010; Pan et al., 2010). Recessive rin mutant tomatoes fail to ripen, as these mutants do not produce high levels of endogenous ethylene and do not respond to exogenous ethylene. TAGL1 is expressed at early and later stages at the onset of ripening. Our results showed that the mRNA level of RIN decreased significantly as compared with the non-treated control fruit (Fig. 6). In fruit treated with sound waves, the expression level of LeHB-1 was reduced after 1 day and 2 days, but increased after 5 days. Furthermore, TAGL1 expression was reduced in the treated fruit, except between 7 and 12 days after sound wave treatment.

Also, CNR and NOR have been reported to regulate the expression of genes related to fruit ripening, including ethylene and carotenoid biosynthesis (Giovannoni, 2004). In this study, the transcript level of CNR decreased at 1–3 days after sound wave treatment, but increased at 7 days after treatment. The expression pattern of CNR was opposite in the sound-treated and non-treated fruit (Fig. 6). Meanwhile, the expression of NOR was very low in sound-treated fruit compared to the non-treated fruit throughout the 14-day observation period (Fig. 6). These results showed that sound wave treatment delayed tomato fruit ripening by inhibiting the expression of some of the most important ethylene biosynthetic and ripening regulatory genes.

This study also showed that the mRNA levels of RIN, ACS2, and ACS4 significantly increased after 7 days in non-treated fruit. However, the expression of these three genes was low, and
remained low up to 14 days after sound wave treatment (Figs. 5 and 6). Martel et al. (2011) showed that RIN binds to the ACS2 and ACS4 promoter, but not to the ACO1 promoter. RIN might be involved in reducing ACS2 and ACS4 expression. Due to the decreased expression of RIN, the expression of ACS2 and ACS4 may have been reduced, but the possibility cannot be ruled out that ACS2 and ACS4 expression is directly controlled by sound waves.

LeHB-1 has been reported to bind to the LeACO1 promoter, which contains the putative HD protein binding sequence, and directly regulates a number of ripening-related genes (Lin et al., 2009). Manavella et al. (2006) showed that transgenic Arabidopsis plants harboring sunflower Hahb-4 exhibited a remarkable delay in senescence and were less sensitive to ethylene. The expression of this gene suppresses the expression of genes associated with ethylene biosynthesis, such as LeACO, and with ethylene signaling, such as ERF2 and ERF5 (Manavella et al., 2006). Blume and Grierson (1997) reported that LeHB-1 is highly expressed in mature green fruit and decreased at the breaker stage, whereas the expression of LeACO1 increased in mature green fruit and accumulated during ripening. These results showed that the level of LeHB-1 transcript declined in the green mature stage and increased after the breaker stage, whereas the expression level of LeACO1 was higher in the early stage of ethylene biosynthesis and was maintained at low levels for up to 14 days after sound wave treatment, indicating that the expression pattern of these genes was affected by the sound wave treatment. As LeHB-1 expression increased, the expression of ACO1 was significantly reduced in sound wave-treated tomato fruit (Figs. 5 and 6).

TAGL1 is known to be involved in ripening in tomato (Itkin et al., 2009; Vrebalov et al., 2009). TAGL1 promotes ripening by activating ACS2, and is reported to be a target of RIN (Itkin et al., 2009; Vrebalov et al., 2009). Silencing of TAGL1 or RIN delays ripening.
Recessive RIN mutant tomatoes fail to ripen, as these mutants do not produce high levels of endogenous ethylene and do not respond to exogenous ethylene. TAGL1 is expressed at early and later stages at the onset of ripening. These real-time RT-PCR results were similar to those reported by others (Busi et al., 2003; Hileman et al., 2006) in non-treated fruit, but TAGL1 expression was reduced during the early stage of ripening in tomato fruit treated with sound waves. Every year, spoilage of postharvest agricultural products results in tremendous economic losses. The purpose of this study was to test the efficacy of delaying the ripening of tomato fruit by controlling the expression of genes related to ethylene biosynthesis through sound treatment. These results propose well that sound wave treatment delays tomato fruit ripening by regulating the expression of ethylene biosynthesis-related genes.

4. Conclusion

Findings in this study lead a conclusion that sound wave can be an external stimulus to delay the ripening of tomato fruit. This study investigated relevant molecular mechanisms through analysis of the expression of ethylene synthesis-related genes, controlling fruit ripening. This study set the stage for further studies of sound wave-mediated responses in plants. Findings of this study indicate that acoustic biology can have viable applications in agriculture and may help to increase product quality and reduce losses. This study also provides the first indication that sound treatment can extend the ripening of tomato fruit. Since this area is still in the beginning stages, more detailed studies need to be performed in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2015.07.015.

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