

Ultrasonic treatment suppresses ethylene signaling and prolongs the freshness of spinach

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ABSTRACT

There have been many studies investigating the application of ultrasonic treatment in vegetables and fruits to eliminate surface contaminants including dirt, microbes, and chemicals such as pesticides. Using a jet ultrasonic washer developed by us to wash food materials, we found that ultrasonic treatment prolonged the freshness of spinach. The stomata closed in the ultrasonicated spinach leaves, whereas those in spinach soaked in water remained open during 24-h storage. Transcriptome analysis revealed that the expression of Ethylene-insensitive 3 Binding F-box protein 1 and 2 (EBF1 and EBF2), which inhibit ethylene signaling, was remarkably increased by ultrasonic treatment, suggesting that the suppression of ethylene signaling allowed stomatal closure in response to abscisic acid signals in the ultrasonicated leaves. Although the precise mechanism of the induction of EBF1 and EBF2 expression by ultrasonic treatment needs to be addressed in further studies, our findings suggest that ultrasonic treatment can be applied to revive and prolong the freshness of leaf vegetables, as well as for their cleaning.

1. Introduction

It typically takes several hours or sometimes days for vegetables and fruits to be transported from the farm to the grocery store, during which time these products pass through the distribution system such as wholesale markets. In the case of leaf vegetables such as spinach, prior to shipment as commercial products, the roots are cut and the leaves begin to wilt with the loss of the water supply upon harvest; further, they are exposed to severe water stress (dry stress) during shelving in the grocery store.

Studies investigating the washing of vegetables and fruits with ultrasonic waves to remove debris and other contaminants have been reported for decades (Lin & Erel, 1992; Zhou et al., 2012; Pinheiro et al., 2015; Alenyorege et al., 2018). Recently, treatment with ultrasonic waves has been combined with other methods, such as sanitizing with chlorine dioxide or modified atmospheric packaging, for microbial surface decontamination and to extend the shelf-life of vegetables and fruits (Salgado et al., 2014; Fan et al., 2019; Wu et al., 2019; Chen et al., 2020).

When harvested vegetables, which are under water stress (dry stress), are soaked in water at the time of washing, they resorb water and recover their freshness. We previously developed a novel jet ultrasonic washer to clean food materials including vegetables, fruits,

meats, fish, and other types of seafood (Sakaguchi, 2013, 2016). In addition to effects such as the removal of pesticide residues, we found that the freshness of ultrasonicated spinach was prolonged compared to spinach soaked simply in water (Sakaguchi & The University of Tokyo, 2017).

To investigate the underlying mechanism of the freshness retention of ultrasonicated spinach, we first examined the behavior of stomata in the ultrasonicated spinach. It has been reported that heat shock reduces water loss in fruits and vegetables to extend the shelf-life, and it is proposed that the induction of heat shock proteins could be involved in these protective effects (Saltveit, 1998; Baloch et al., 2006; Rico et al., 2007). It is possible that the heat shock response is induced in the ultrasonicated spinach, since the mechanical energy of the ultrasonic vibrations changes into thermal energy due to the viscoelasticity of water. In this study, transcriptome analysis was conducted to reveal the molecular responses induced in ultrasonicated spinach.

2. Materials and methods

2.1. Ultrasonic treatment

We previously designed and produced a jet ultrasonic washer that was used to wash spinach in this study. An image and a diagram of the

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ultrasonic washer are presented in Fig. 1. Details of this equipment are described in Utility Model Registration of Japan No. 3187858 (Sakaguchi, 2013) and Japan Patents JP5863557 (Sakaguchi, 2016) and JP6095087 (Sakaguchi & The University of Tokyo, 2017). The jet ultrasonic washer is equipped with a cylindrical washing tank 200 mm in diameter, with a water depth of 120 mm. The power consumption of the unit is 100 W and the volume of water in the washing tank is 3.77 L. The washer is continuously supplied with fresh clean water (tap water) from the water supply port (at the bottom center of the washing tank) at a flow velocity of 0.5–2.0 L/min. The ultrasonic intensity (3–20 psi) was measured with the Sonic Meter SM-1000 (Shinka Industry Co., Ltd., Kawasaki, Japan). In this study, the washing tank was filled with tap water and the spinaches were allowed to soak while being subjected to ultrasonic treatment at 40 kHz for 15 min. Then, the spinaches were removed from the washing tank, drained for 5 min and kept in sealed plastic containers at 4 °C or ambient temperature (15 °C). After 21-h storage, stomata on the dorsal surface of leaves were microscopically observed and the width and length of the stomatal aperture were measured and the stomatal aperture index was calculated. At the same time, the spinaches were soaked in tap water for 15 min without ultrasonic treatment as controls.

2.2. Spinach

The spinach used in the experiments was purchased at a grocery store near Kashiwa Campus, The University of Tokyo (Kashiwa City, Chiba Prefecture, Japan), and obviously wilted spinach was not used. The freshness of spinach is closely dependent on a variety of factors such as handling, transportation and storage conditions, and time after harvest. Therefore, we purchased several bunches of spinach at the same time and divided them into two groups at random, one was washed with ultrasonic waves and the other was simply soaked in water, to minimize quality differences among the spinach samples in each experiments.

2.3. Transcriptome analysis

Six bunches of spinach were treated with ultrasonic waves (40 kHz, 15 min) with the jet, then drained and kept in sealed plastic containers at 4 °C in a refrigerator. After 3 or 24 h, one leaf with petiole was isolated from each of the 6 bunches, immediately frozen between blocks of dry ice (1 kg), and powdered in a chilled laboratory mortar with a pestle. A 1 g portion of the frozen powder was used for extraction of total RNA (100–200 µg each) with TRIsure (Nihon Genetics, Tokyo, Japan). Total RNA (1 µg) was subjected to RNA-seq analysis with NovaSeq 6000 (Illumina, San Diego, CA, USA). The obtained reads were mapped to the genome assembly of spinach in NCBI (ASM200726v1, https://www.ncbi.nlm.nih.gov/assembly/GCF_002007265.1) and gene annotation was conducted with *Spinacia*

oleracea Annotation Release 100 by NCBI. Six bunches of spinach soaked in water for 15 min without ultrasonic treatment were subjected to extraction of total RNA and analyzed in the same manner as above. In this study, RNA-seq analysis was conducted once for each RNA sample extracted from control, ultrasonicated, or soaked only spinach leaves at 3 and 24 h after treatment (5 RNA-seq analyses in total). Fold changes (FC) were calculated using edgeR (Robinson et al., 2010) and genes with FC > 1.5 were selected as differentially expressed genes (DEGs). The BioProject ID is PRJDB10558 and the DRA accession ID is DRA010883. The accession number of each read is DRR248963, DRR248964, DRR248965, DRR248966, DRR248967 for the control, 3 h after soaking only, 24 h after soaking only, 3 h after ultrasonication, and 24 h after ultrasonication, respectively.

3. Results

3.1. Ultrasonic washing revived freshness of spinach

Spinach samples were washed with ultrasonic waves for 15 min and then stored in sealed plastic containers to prevent desiccation at ambient temperature (20 °C) for 8 days. The leaves of the ultrasonicated spinach spread widely and showed less wilt than those soaked in water only (Fig. 2).

3.2. Ultrasonic treatment induced stomatal closure in spinach

We hypothesized that ultrasonic treatment regulates the opening/-closing of stomata in spinach. Spinach leaves were soaked in water or washed with ultrasonic waves for 15 min and kept in sealed containers in the refrigerator for 3 h, and then the stomata were observed under a microscope. Almost all stomata were open in both the sample soaked in water only and that treated with ultrasonic waves. When the leaves were left for 21 h at 4 °C in a sealed container and again examined microscopically, we found that almost all stomata were open in the soaked leaves (Fig. 3A), while almost all stomata were closed in the leaves ultrasonically treated (Fig. 3B).

3.3. Transcriptome analysis revealed *EBF1* and *EBF2* were induced in ultrasonicated spinach

To verify the contribution of heat shock proteins in “refreshing” the ultrasonicated spinach, we conducted transcriptome analysis in spinach. We found that most genes were expressed similarly in the ultrasonicated and water-soaked spinach. At 3 h after treatment, the expression of 51 genes decreased in the spinach soaked in water but increased in the ultrasonicated spinach (Table 1, Supplementary Table S1). Further, the expression of 42 genes increased in the water-soaked spinach but decreased in the ultrasonicated spinach

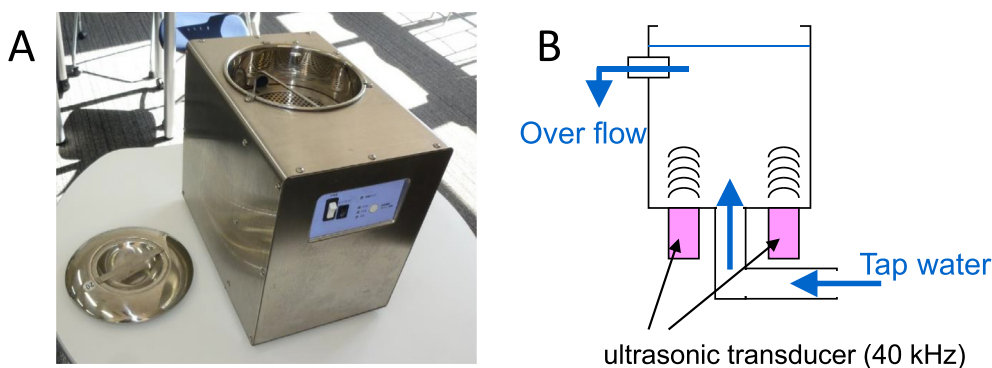


Fig. 1. An image (A) and a schematic diagram of the working principle (B) of the jet ultrasonic washer used in this study. Clean tap water is supplied into the cylindrical washing tank from the bottom of the tank during ultrasonic treatment.

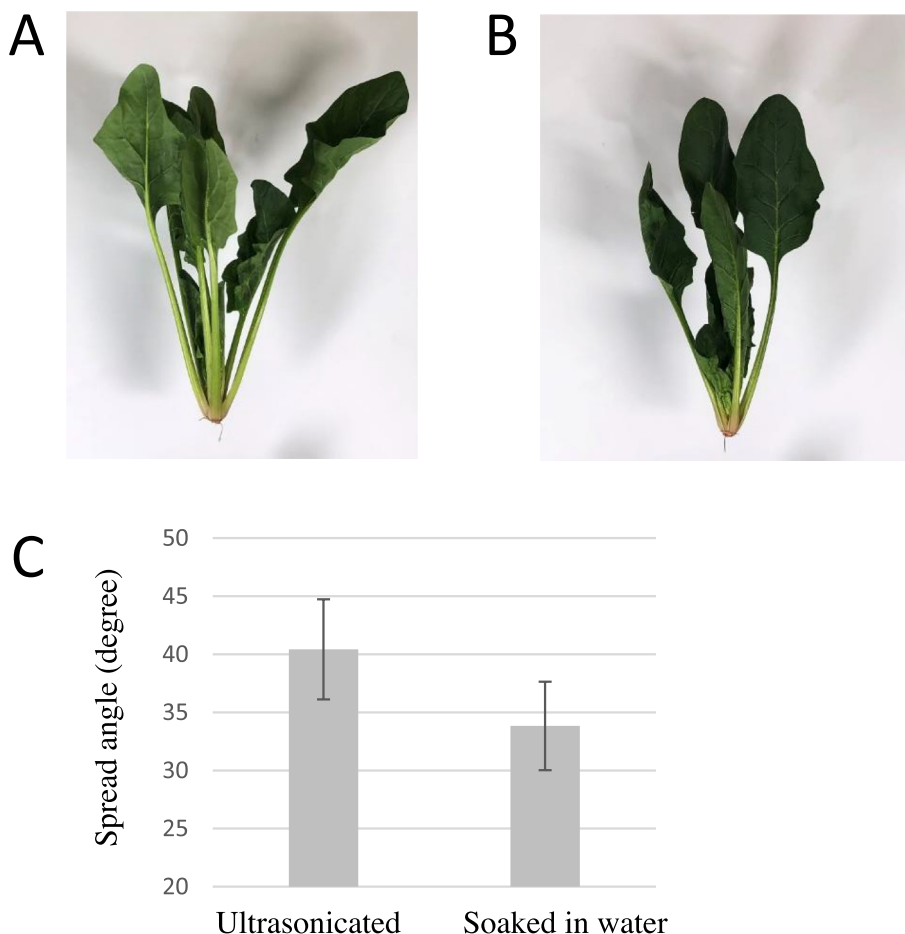


Fig. 2. Images of ultrasonicated (A) and water-soaked (B) spinach bunches after 8-day storage in sealed containers. Bunches were hung with a fishing line and a small hook, and images were taken and presented upside down. The angle of spread of each bunch was measured and is presented as mean \pm SD ($n = 7$, $p < 0.05$) (C).

(Supplementary Table S2). At 24 h after treatment, the expression of 44 genes decreased in the spinach soaked in water and increased in the ultrasonicated spinach (Table 2, Supplementary Table S3), and the expression of 48 genes increased in the water-soaked spinach and decreased in the ultrasonicated spinach (Supplementary Table S4). No heat shock proteins were identified in the genes showing increased expression in the ultrasonicated spinach in this study.

It is well known that abscisic acid and ethylene control the opening and closing of stomata (Zhu, 2002; Tanaka et al., 2005; She & Song, 2012). In transcriptome analysis of the spinach 3 h after soaking in water, the gene expression of abscisic acid synthesizing enzyme (LOC110805680) decreased and that of abscisic acid degrading enzyme (LOC110788345) increased, indicating the inactivation of abscisic acid signaling. These changes were similar in the ultrasonicated spinach (Table 3, Supplementary Table S5, S6). It is possible that the spinach leaves and petioles resorbed water when soaked or ultrasonicated, and abscisic acid signaling might be weakened because of the reduced water stress. In the spinach soaked in water and stored for 24 h, gene expression of both abscisic acid synthesizing enzyme (LOC110805680) and abscisic acid degrading enzyme (LOC110788345) further decreased. In the ultrasonicated spinach stored for 24 h, gene expression of abscisic acid degrading enzyme (LOC110788345) decreased as well, while that of abscisic acid synthesizing enzyme (LOC110805680) did not, suggesting that abscisic acid signaling was re-activated 24 h after the ultrasonic treatment. RAB18 (LOC110776340) is one of the stress-responsive genes encod-

ing the glycine-rich dehydrin protein, which is expressed in the guard cells of stomata, and its expression is induced by abscisic acid (Nylander et al., 2001; Tanaka et al., 2005). Expression of RAB18 decreased 3 h after treatment and increased 24 h after treatment in both the soaked and the ultrasonicated leaves (Table 3, Supplementary Tables S5, S6), strongly suggesting that the leaves were again under water stress after 24-h storage and abscisic signaling was reactivated.

Next, we focused on the expression of genes involved in ethylene synthesis. Gene expression of ACC synthase (LOC110805602, LOC110805592) and ACC oxidase (LOC110777334, LOC110803795) (Yang & Hoffman, 1984; Johnson & Ecker, 1998) increased 3 h after treatment in both the soaked and the ultrasonicated spinach. Expression of ACC synthase (LOC110805602, LOC110805592) decreased 24 h after treatment, but expression of ACC oxidase (LOC110777334, LOC110803795) was maintained in both the soaked and the ultrasonicated spinach (Table 3, Supplementary Tables S5, S7); thus, ultrasonic treatment did not affect the expression of ACC synthase (LOC110805602, LOC110805592) and ACC oxidase (LOC110795784, LOC110777334, LOC110803795). Interestingly, Ethylene-insensitive 3 Binding F-box protein 1 (EBF1, LOC110788382) was identified as a DEG with a large read count, and its expression was decreased in the soaked spinach but increased in the ultrasonicated leaves 3 h after treatment. Further, the expression of EBF1 was remarkably increased in the ultrasonicated spinach 24 h after treatment. The expression of Ethylene-insensitive 3 Binding F-

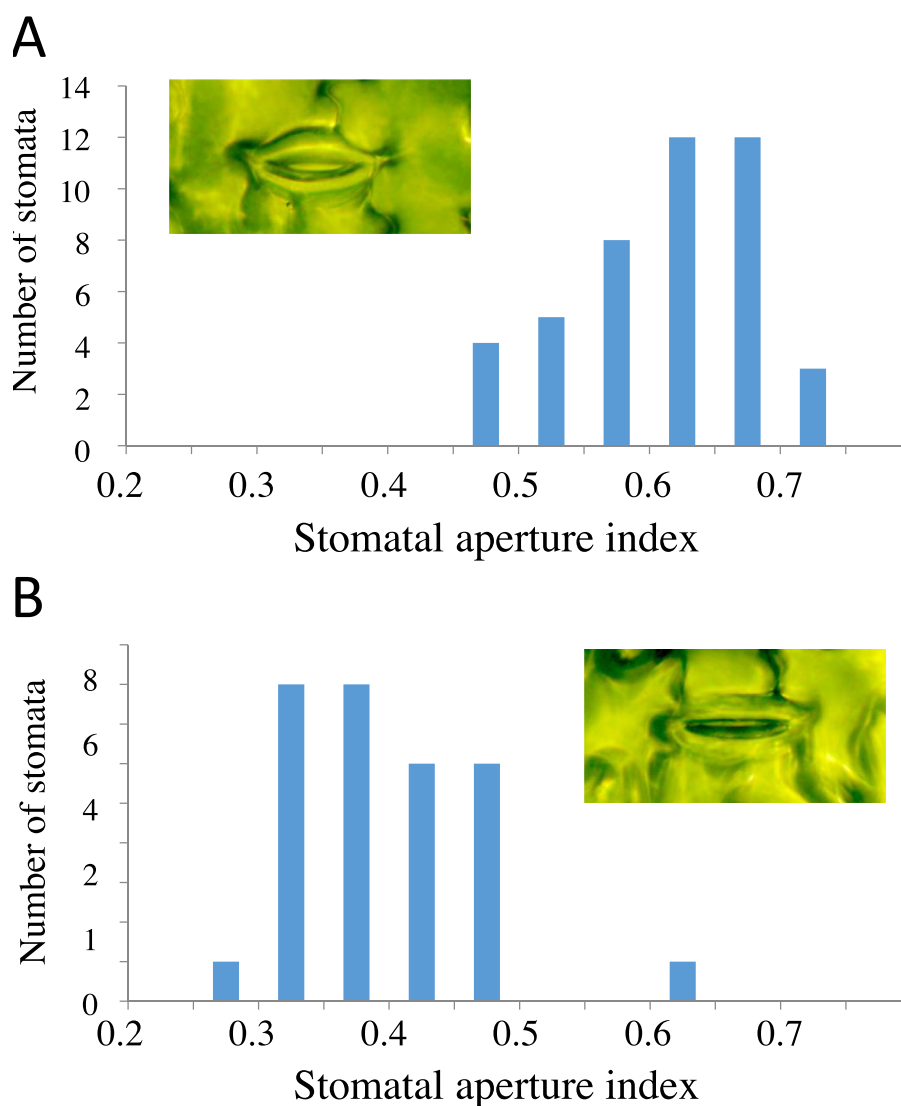


Fig. 3. Effect of ultrasonic treatment on stomatal opening in spinach leaves. Spinach leaves were ultrasonicated or soaked in water for 15 min and after 21-h storage stomata on the dorsal surface of leaves were microscopically observed. Histograms of stomatal aperture index in the soaked leaves (A) and the ultrasonicated leaves (B) are presented. The inset images in A and B are an open stoma in a soaked leaf and a closed stoma in an ultrasonicated leaf, respectively.

Table 1

Effect of ultrasonic treatment on gene expression in spinach 3 h after the treatment.

	Down in Sonicated	No change in sonicated	Up in sonicated
Dow in Soaked	835	546	51
No change in soaked	1086	13,471	881
Up in soaked	42	698	1600

Note: 19,210 genes were annotated.

box protein 2 (EBF2, LOC110796826) was increased 3 h after treatment and remarkably increased 24 h after treatment in both the soaked and ultrasonicated spinach (Table 3, Supplementary Tables S5, S7).

Gene expression related to the other major plant hormones such as cytokinin, gibberellin, and auxin was similar in the soaked and ultrasonicated spinach (Supplementary Tables S8, S9, S10). No noteworthy changes associated with the ultrasonic treatment were found for p450 gene expression (Supplementary Table S11), which includes the gibberellin synthesizing pathway genes (Pimenta Lange & Lange, 2008),

Table 2

Effect of ultrasonic treatment on gene expression in spinach 24 h after the treatment.

	Down in sonicated	No change in sonicated	Up in sonicated
Down in Soaked	2776	887	44
No change in soaked	856	10,403	671
Up in soaked	48	723	2820

Note: 19,228 genes were annotated.

suggesting that these were not strongly impacted by ultrasonic treatment.

4. Discussion

Using a jet ultrasonic washer that we developed, the freshness of spinach leaves was revived and prolonged after washing with ultrasonic waves. Heat shock treatment has been widely applied to maintain the freshness and extend the shelf-life of harvested fresh

Table 3

Effects of ultrasonic treatment on expression of abscisic acid and ethylene signaling related genes.

Gene_Symbol	Description	DIP_3H/ Control.fc	SONIC_3H/ Control.fc	DIP_24H/ Control.fc	SONIC_24H/ Control.fc	SONIC_3H/ DIP_3H.fc	SONIC_24H/ DIP_24H.fc
LOC110805680	Absciscic acid synthesizing enzyme 9-cis-epoxycarotenoid dioxygenase NCED1, chloroplastic	−3.605	−4.247	−8.129	−4.211	−1.178	1.931
LOC110788345	Absciscic acid degrading enzyme abscisic acid 8'-hydroxylase 1-like	1.573	1.691	−1.721	−1.832	1.075	−1.065
LOC110776340	dehydrin Rab18-like	−1.461	−1.639	4.560	4.576	−1.122	1.004
LOC110805602	ACC synthase 1-aminocyclopropane-1-carboxylate synthase-like	2.272	1.812	−4.984	−2.592	−1.253	1.923
LOC110805592	1-aminocyclopropane-1-carboxylate synthase-like	2.247	1.778	−5.312	−2.579	−1.263	2.060
LOC110795784	ACC oxidase 1-aminocyclopropane-1-carboxylate oxidase homolog 4-like	1.015	1.161	−1.127	1.008	1.144	1.137
LOC110777334	1-aminocyclopropane-1-carboxylate oxidase- like	1.563	1.591	1.944	2.193	1.018	1.128
LOC110803795	1-aminocyclopropane-1-carboxylate oxidase 4-like	1.812	1.830	1.815	1.510	1.010	−1.202
LOC110788382	EIN3-binding F-box protein 1-like (EBF1)	−1.584	1.841	1.434	2.635	2.917	1.837
LOC110796826	EIN3-binding F-box protein 2-like (EBF2)	1.619	10.632	10.134	18.325	6.566	1.808

Note: Gene symbol: Gene ID in the NCBI database (<https://www.ncbi.nlm.nih.gov/>); DIP_3H/Control.fc: fold change of the soaked leaves 3 h after the treatment against control (no treatment); SONIC_3H/Control.fc: fold change of the ultrasonicated leaves 3 h after the treatment against control (no treatment); DIP_24H/Control.fc: fold change of the soaked leaves 24 h after the treatment against control (no treatment); SONIC_24H/Control.fc: fold change of the ultrasonicated leaves 24 h after the treatment against control (no treatment); SONIC_3H/DIP_3H.fc: fold change of the ultrasonicated leaves 3 h after the treatment against the soaked leaves; SONIC_24H/DIP_24H.fc: fold change of the ultrasonicated leaves 24 h after the treatment against the soaked leaves.

vegetables and fruits, as heat shock proteins might be induced and protective (Saltveit, 1998; Baloch et al., 2006; Rico et al., 2007; Ansorena et al., 2014). At first, we hypothesized that ultrasonic washing would heat the spinach and induce heat shock responses; however, transcriptome analysis strongly suggested that the expression of heat shock proteins was not promoted in the ultrasonicated spinach.

Post-harvest vegetables undergo water stress and the abscisic acid pathway is strongly activated (Bray, 1997; Zhu, 2002). The activation of the abscisic acid degrading enzyme in the leaves 3 h after water soaking indicates the mitigation of water stress. Gene expression related to ethylene synthesis (Yang & Hoffman, 1984; Kevin et al., 2002) showed no remarkable changes during storage, suggesting that a small amount of ethylene might be synthesized continuously during 24-h storage after treatment. On the other hand, expression of RAB18 was remarkably increased in both the soaked and the ultrasonicated leaves. Since RAB18 expression is strongly dependent on abscisic acid signaling and is used as an index of active abscisic acid signaling (Nylander et al., 2001; Tanaka et al., 2005), we can assume that abscisic acid signaling was re-activated during 24-h storage after soaking in water. The expression of abscisic acid synthase did not decreased in the ultrasonicated leaves after 24-h storage. In contrast, soaked leaves showed decreased expression of abscisic acid synthase after 24-h storage, suggesting that abscisic acid signaling was less activated in the water-soaked leaves.

In the ultrasonicated leaves, EBF1 and EBF2 gene expression increased remarkably during storage after treatment (Table 3). Ethylene, which controls germination, flower bud formation, fruit maturation, and plant aging (Khan et al., 2017; Dubois et al., 2018), is one of the major plant hormones, and EBF1 and EBF2 strictly regulate the physiological actions of ethylene (Guo & Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Yang et al., 2010). Both EBF1 and EBF2 bind EIN3, which is the major transducer in ethylene signaling, resulting in EIN3 ubiquitination and subsequent proteolysis by the proteasome system (Guo & Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). The weak expression of EBF1 and EBF2 in the soaked leaves may result in quality deterioration such as ethylene-induced

aging, in addition to water loss. It is reported that EBF2 expression is induced by ethylene in *Arabidopsis* (Potuschak et al., 2003; Konishi & Yanagisawa, 2008); however, the mechanism of the induced expression of EBF1 has not yet been elucidated. Although there is overlap in the functions of EBF1 and EBF2, EBF1 responds to lower concentrations of ethylene, while EBF2 responds to higher concentrations (Binder et al., 2007). In the soaked spinach, EBF1 and EBF2 were weakly expressed and the continuous production of ethylene might inhibit the closing of stomata by abscisic acid.

Various antagonistic or reciprocal physiological interactions are known between abscisic acid and ethylene signaling (Beaudoin et al., 2000; Schroeder et al., 2001; Dodd, 2003; Li & Huang, 2011). Absciscic acid signaling is activated by the lack of water to promote stomatal closure, while ethylene has inhibitory effects (Tanaka et al., 2005; She & Song, 2012). In this study, we observed that ultrasonic treatment remarkably increased the expression of EBF1 and EBF2. Ultrasonic treatment could suppress ethylene signaling via EBF1 and EBF2 and allow stomatal closure by abscisic acid, thereby avoiding water loss through the stomata. In contrast, the weak expression of EBF1 and EBF2 may allow ethylene to prevent stomatal closure by abscisic acid. In this scenario, the spinach leaf might rapidly lose water after water soaking and wilt, in addition to the aging promoting action of ethylene.

High-intensity ultrasound removes and/or kills microbes on the surface of vegetables and fruits, and has been widely used to decontaminate and extend the shelf-life of products (Bilek & Turantaş, 2013). It has also been reported that high-intensity ultrasound disrupts cells and tissues, inactivating enzymes responsible for browning and texture loss, and prolonging the shelf-life (Jambrak et al., 2007; Amaral et al., 2015). In this study, we observed that low-intensity ultrasound revives the freshness of spinach and can prolong its shelf-life. In recent years, evidence has been accumulating that ultrasonic treatment has physiological and biochemical effects on plants (Yu et al., 2016; Ding et al., 2018a, 2018b; Wang et al., 2019). Specifically, it has been reported that ultrasonic treatment promotes germination and increases γ -aminobutyric acid (GABA) content (Yang et al.,

2015; Ding et al., 2018a, 2018b). It is likely that ultrasonic treatment modulates ethylene and abscisic acid signaling in seeds and promotes germination in some plant species (Johnson & Ecker, 1998; Vishal & Kumar, 2018).

The molecular mechanism of the promotion of EBF1 and EBF2 expression by ultrasonic treatment needs to be elucidated in future studies. Since the spinach genome remains poorly characterized, it was not possible to conduct detailed transcriptome analysis to elucidate the molecular mechanisms of the biological effects of ultrasonic treatment. Thus, detailed molecular biological analysis in a well-studied model plant such as *Arabidopsis* might assist in elucidating the precise molecular events induced by ultrasonic treatment in plants. In humans, ultrasound has been used as a therapeutic tool to stimulate osteoblast differentiation (Macione et al., 2015); however, the precise mechanisms of the biological effects of ultrasound are almost completely unknown. In biochemistry laboratories, high-intensity ultrasonic treatment has been widely used to disrupt cells or tissues in preparing samples for biochemical experiments. On the other hand, the effects of low-intensity ultrasonic treatment on living cells and tissues have not been investigated, representing a new frontier for future exploration.

5. Conclusions

The freshness of spinach washed using a jet ultrasonic washer was revived and prolonged. Ultrasonic treatment prompted stomatal closing in spinach leaves and transcriptome analysis revealed that expression of Ethylene-insensitive 3 Binding F-box protein 1 and 2 (EBF1 and EBF2) was induced, which suppresses ethylene signaling. During storage after ultrasonic treatment, spinach leaves exhibited water stress and abscisic acid signaling was re-activated to close stomata. In contrast, in water-soaked leaves, the expression of EBF1 and EBF2 was weak and the stomata did not close, resulting in rapid wilting compared to ultrasonicated leaves during storage. Although the precise mechanism of the induction of EBF1 and EBF2 expression by ultrasonic treatment should be addressed in further studies, our findings suggest that ultrasonic treatment induces some physiological responses in spinach and is applicable for prolonging the freshness of spinach as well as cleaning the produce.

CRedit authorship contribution statement

Shoji Oda: Conceptualization, Investigation, Data curation, Writing - original draft, Writing - review & editing, Supervision. **Masaaki Sakaguchi:** Conceptualization, Resources, Methodology, Investigation, Project administration. **Xiesong Yang:** Investigation. **Qinyao Liu:** Investigation. **Kohei Iwasaki:** Project administration, Writing - original draft. **Kaori Nishibayashi:** Conceptualization, Writing - original draft, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Masaaki Sakaguchi and Shoji Oda are employed by The University of Tokyo, which owns the Japan patent (JP6095087). Masaaki Sakaguchi owns the Japan patent (JP5863557 and JP6095087). Masaaki Sakaguchi owns the Japan Utility Model Registration No. 3187858.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochms.2021.100026>.

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