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Original Article

Induction of resistance to diseases in plant by aerial ultrasound irradiation

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Ultrasound, which refers to frequencies above the audible limit of human hearing, is a candidate for inducing resistance to pathogens in plants. We revealed that aerial ultrasound of 40.5 kHz could induce disease resistance in tomatoes and rice when the plants were irradiated with ultrasound of *ca.* 100 dB for 2 weeks during nursery season and reduced the incidence of Fusarium wilt and blast diseases, respectively, when plants were inoculated with pathogen 0 or 1 week after terminating irradiation. Disease control efficacy was also observed with ultrasound at frequencies of 19.8 and 28.9 kHz. However, cabbage yellows and powdery mildew on lettuce were not suppressed by ultrasound irradiation. No significant positive or negative effect on growth was observed in tomato and rice plants. RT-qPCR showed that the expression of *PR1a* involved in the salicylic acid (SA) signaling pathway was upregulated in the ultrasound-irradiated tomato.

Keywords: ultrasound, physical control, SA signaling pathway, soilborne fusarium disease, blast, powdery mildew.

Electronic supplementary material: The online version of this article contains supplementary material (Supplemental Figs. S1–S2), which is available at http://www.jstage.jst.go.jp/browse/jpestics/

Introduction

Plants are continuously challenged by a wide spectrum of environmental stimuli. These include extreme temperature, drought, wind, vibration, pests, and microbes. Plants can appropriately recognize these stimuli and respond to them.

Plants usually protect themselves from microbes by activating defense reactions after recognizing microbial stimuli. However, some microbes, designated pathogens, are able to weaken or invalidate the defense to invade plants. Therefore, activating the defense prior to the approach of pathogenic microbes seems to be a good way to control the diseases caused by the pathogens.

Interestingly, plants' response pathways to abiotic and biotic stimuli partly overlap.[1)] Thus, when plants are exposed to abiotic stimuli, such as heat, wind, drought, or vibration, physiological changes caused by biotic stimuli can occur by chance.[2)] For example, chemical stimuli, such as probenazole (PBZ), validamycin A (VMA), acibenzolar-*S*-methyl (ASM), tiadinil (TDL), isotianil, and fosetyl, have been used as plant activators that do not have direct antipathogen activity but induce disease resistance in plants. All of these plant activators are reported to induce systemic acquired resistance (SAR) mediated by the salicylic acid (SA) signaling pathway (Fig. S1).[3,4)]

Light, one mechanical stimulus, is used to induce disease resistance in plants. For example, the irradiation of plants with a green LED of *ca.* 550 nm has been put to practical use under the name of "Midorikikuzou" (Shikoku Research Institute), which can suppress strawberry (*Fragaria*×*ananassa*) anthracnose caused by *Colletotrichum acutatum*.

There are several reports on using sound vibration, another mechanical stimulus, to generate physiological change in plants.

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For instance, "green music" which comprises natural sounds, such as songs of birds and insects, increased the seed germination rate in zucchini (*Cucurbita pepo*) and okra (*Abelmoschus esculentus*)[5] and enhanced the uptake of polyamine and oxygen in Chinese cabbage (*Brassica campestris*) and cucumber (*Cucumis sativus*).[6] Root elongation toward the 220 Hz sound source was observed in corn (*Zea mays*).[7] Furthermore, indole acetic acid accumulated in *Chrysanthemum* spp. callus when irradiated with 1.4 kHz sound, while abscisic acid decreased.[8] Recently, Choi *et al.* (2017)[9] reported that 10 days (3 hr/day) of irradiation with 1 kHz sound primed SAR and increased resistance to *Botrytis cinerea* infection in *Arabidopsis thaliana*. However, few reports have described the induction of resistance to diseases, especially in edible plants by sound irradiation.

Despite a number of studies on ultrasound,[10–13] no report has examined the physiological effects of ultrasound on plants. Ultrasound waves have frequencies higher than the upper audible limit (usually *ca.* 20 kHz) of human hearing. In this report, we found that ultrasound induces plant immunity against pathogens.

Materials and Methods

1. Plant materials and growth conditions

Tomato (*Solanum lycopersicum* cvs. Momotaro, Takii & Co., and Moneymaker, Baker Creek Heirloom Seeds), cabbage (*Brassica oleracea* var. *capitata* cv. Shikidori, Takii & Co.), and rice (*Oryza sativa* cvs. Aichi-asahi and Kinuhikari) were used. Plants were grown in a greenhouse (20–35°C) or in a growth chamber (25°C, light : dark = 16 hr : 8 hr) in sterilized soil (JA Nippi Engei Baido, Nihon Hiryo).

2. Aerial ultrasound

An aerial ultrasound oscillator with a frequency of 40.5 kHz was developed and used. The oscillation pattern was composed of intermittent pulse waves (Fig. 1a). The pulse width is 7 msec, and the pulse frequency repeatedly shifts. Pulse waves with the higher pulse frequency were oscillated with the lower sound pressure level. The sound pressure level inside a circle 50 cm in diameter was maintained at over 100 dB at a distance of 70 cm from the oscillator. The oscillator was set over the plants at a distance of 70 cm, and plants were irradiated with aerial ultrasound starting for 1- or 2-week-old plants and continuing for 1–2 weeks (24 hr a day), as shown in Fig. 1b, in a soundproof area covered with acrylic boards or PVC (polyvinyl chloride) sheets (Fig. 1c). Oscillators of 19.8 and 28.9 kHz ultrasound were also prepared to study the difference in effect depending on the frequencies.

3. Fungal isolates, cultural conditions, and inoculation testing

Inoculation with pathogens was done 0 or 1 week after the termination of ultrasound irradiation.

The tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici* JCM 12575 (*Fol*) and the cabbage yellows fungus *F. oxysporum* f. sp. *conglutinans* Cong:1-1 (*Foc*) were cultured for 3–5 days on a rotary shaker at 120 rpm and 25°C in potato sucrose broth (PSB)

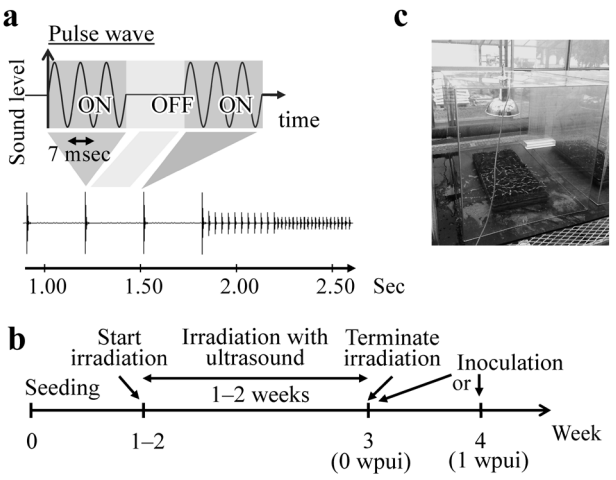

Fig. 1. Aerial ultrasonic conditions and an experimental flow. Seedlings of each plant were irradiated with ultrasound of 40.5 kHz frequency with *ca.* 100 dB sound pressure level. The oscillation pattern was composed of intermittent pulse waves. The pulse width is 7 msec, and the pulse frequency repeatedly shifts. The intermittent pulse waves are illustrated (a). Irradiation with ultrasound started when plants were 1 week old, continued for 1–2 weeks (24 hr a day), and plants were inoculated with pathogens at 0 or 1 week after terminating ultrasound irradiation (b). Tomato seedlings irradiated with aerial ultrasound in a limited irradiation area (50 cm diameter at 70 cm from the oscillator) in a sound-proof area (c).

medium. Bud cells were collected by centrifugation (1630×*g*, 10 min), suspended (1.0×10^7 bud cells/mL for *Fol* or 1.0×10^5 bud cells/mL for *Foc*) in sterilized distilled water, and used as inocula. Each inoculum of *Fol* or *Foc* was poured (1 mL/100 g soil) into the soil for inoculation. To promote pathogen infection, plant roots were injured beforehand by inserting a plastic peg 5 times/plant into the soil. The severity of the disease of each tomato or cabbage plant was evaluated approximately 40 or 14 days after inoculation with *Fol* or *Foc*, respectively. Each plant's symptoms were indexed following the precedent of Inami *et al.* (2014)[14] or Kashiwa *et al.* (2013)[15]: 0, no symptoms; 1, lower leaves yellowing; 2, lower and upper leaves yellowing; 3, lower leaves yellowing and wilting, and upper leaves yellowing; 4, all leaves wilting and yellowing, or dead.

The tomato powdery mildew fungus, *Oidium* sp., was maintained on tomato plants in a greenhouse. Tomato plants presenting signs of powdery mildew were used as an inoculum by placing among the tested tomato seedlings. Two weeks after starting inoculation, the disease incidence of each plant was evaluated as ranging from 0 to 4, following the indexes: 0, no symptoms; 1, a foliar area of 0–5% indicating powdery mildew symptoms; 2, 6–30%; 3, 31–60%; and 4, 61–100%.

The rice blast fungus, *Pyricularia oryzae* Hoku 1 (*Po*), was cultured for 14 days at 25°C on an oatmeal agar medium plate. Aerial hyphae were removed by scraping with an autoclaved spatula, and the plate was irradiated with blue light of a blacklight (368 nm; FL20S·BL-B, Hitachi) for 3 days to stimulate conidial formation. The formed conidia were recovered using an autoclaved spatula and sterilized distilled water, and the conidial

suspension was adjusted to 2.0×10^4 conidia/mL and used as an inoculum. The conidial suspension was sprayed onto rice leaves (*ca.* 0.2 mL/plant) using airbrush (PC-308, Olympos). After inoculation, plants were maintained in a humidified growth chamber at 27°C and 100% humidity in the dark for 24 hr and kept in a 25–28°C greenhouse for a week to promote infection and symptom development. The number of lesions on the upper three leaves of each rice plant was counted 1 week after inoculation with *Po*.[16]

All statistical analyses were performed with R and EZR softwares.[17]

4. RNA extraction and quantitative real-time PCR (RT-qPCR)

After the termination of 2 weeks of irradiation with ultrasound (40.5 kHz, *ca.* 100 dB and intermittent pulse wave), tomato (cv. Moneymaker) plants were inoculated with *Fol* as mentioned above. Total RNA was extracted from tomato leaves, stems, and roots, separately, at 0 and 24 hr post inoculation (hpi) using Trizol reagent in accordance with the manufacturer's instructions (Invitrogen). Total RNA was treated with RQ1 DNase (Promega), and cDNA was generated using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR was performed using GoTaq qPCR Master Mix (Promega) and Thermal Cycler Dice Real Time System II MRX [TP960] (Takara Bio Inc.). The relative expression levels of mRNA were determined by normalizing the PCR threshold cycle number of each gene with that of the *Actin2* (Solyc11g005330) reference gene as the standard. Two or three biological replicates were used in each treatment. The primers used for RT-qPCR are shown in Table 1.[18,19]

Results and Discussion

1. Wilt was suppressed in tomato plants irradiated with 40.5 kHz aerial ultrasound before inoculation

We examined whether aerial ultrasound irradiation induces disease resistance in tomatoes and suppresses tomato wilt caused by *Fol*. We irradiated tomato seedlings with aerial ultrasound (40.5 kHz, *ca.* 100 dB, intermittent pulses; Fig. 1a) for 2 weeks before inoculation. As the ultrasound irradiation was terminated prior to inoculation, the pathogen was not directly affected by the ultrasound.

Wilt was significantly suppressed in tomato plants irradiated for 2 weeks with ultrasound as compared to non-irradiated plants when they were inoculated with *Fol* just after the termination of ultrasound irradiation (0 weeks after the termination of ultrasound irradiation, 0 wpui; Fig. 2a). The disease was also suppressed in tomato plants irradiated for 2 weeks with ultrasound when they were inoculated with *Fol* 1 week after the termination of ultrasound irradiation (1 wpui; Fig. 2b). As the disease prevention effect of ultrasound irradiation lasted longer than 1 week after irradiation was terminated, the ultrasound irradiation seemed to induce disease resistance in tomatoes.

One-week irradiation also conferred a wilt control effect, but

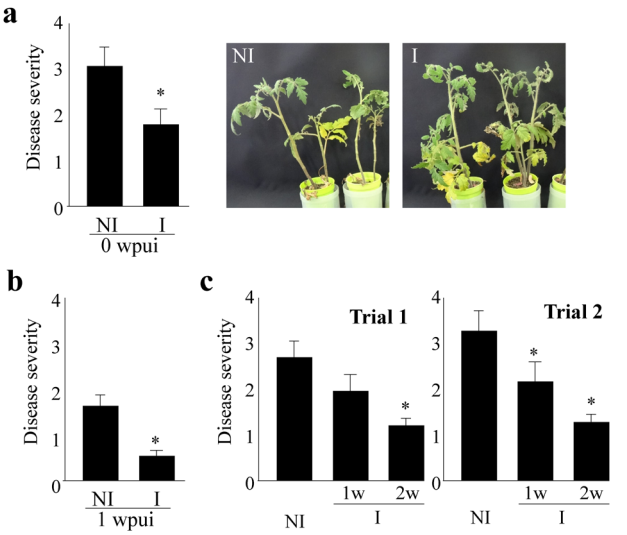

Fig. 2. Wilt was suppressed in tomato plants irradiated by aerial ultrasound before inoculation. One-week-old tomato seedlings were irradiated with 40.5 kHz ultrasound for 1 or 2 weeks (I) or without irradiation (NI) and inoculated with the tomato wilt pathogen *F. oxysporum* f. sp. *lycopersici* (*Fol*) 0 or 1 week after terminating ultrasound irradiation. About 40 days after inoculation, the severity of each plant's disease was evaluated from 0 to 4 following the indexes: 0, no symptoms; 1, lower leaves yellowing; 2, lower and upper leaves yellowing; 3, lower leaves yellowing and wilting and upper leaves yellowing; 4, all leaves wilting and yellowing or dead. Two-week ultrasound-irradiated tomato plants (cv. Moneymaker) presented significant wilt suppression when they were inoculated with *Fol* just after the termination of ultrasound irradiation (0 wpui) (a). Two-week ultrasound-irradiated tomato plants (cv. Moneymaker) presented wilt suppression when they were inoculated with *Fol* 1 week after the termination of ultrasound irradiation (1 wpui) (b). One week (1w) of irradiation also conferred a wilt control effect in cv. Moneymaker when plants were inoculated with *Fol* 1 week after the termination of ultrasound irradiation, but the effect of 2-week (2w) irradiation was higher (c). The severity of disease was analyzed with R, Wilcoxon rank sum test. $p<0.05$, bars = standard error, Asterisk means significance as compared to NI.

Table 1. List of primers used for RT-qPCR

Target gene	Accession number	Forward primer 5′→3′	Reverse primer 5′→3′	Amplification size	Reference
PAL1	M83314	TCCAGTGACTAACCATGTCCAAAG	AAGAGCCACGAGATAGGTTGATG	136 bp	This study
NIM1	AY640378	GCTCCAGGCGGTAAGGAAA	GGCATCAAAACTCACCTCATACTC	147 bp	This study
PR1a	M69247	AGACTATCTTGCGGTTCACAACG	TCCCCAGCACCAGAATGAA	144 bp	This study
LoxD	U37840	GACTGGTCCAAGTTCACGATCC	ATGTGCTGCCAATATAAATGGTTCC	178 bp	Uppalapati *et al.* 2005[18]
Actin2	Solyc11g005330	TTGCTGACCGTATGAGGAAG	GGACAATGGATGGACCAGAC	186 bp	Du *et al.* 2017[19]

* Functions of each gene are shown in Fig. S1.

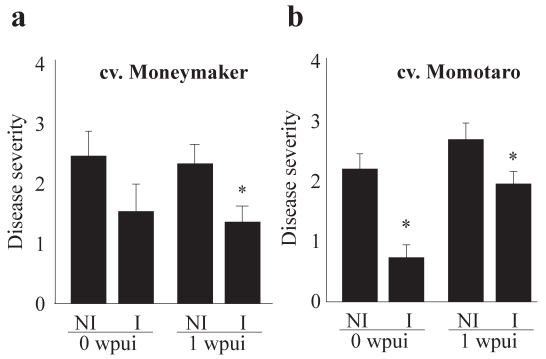

Fig. 3. Wilt was suppressed in tomato plants irradiated by aerial ultrasound using the traveling device where the total period of irradiation of each plant by ultrasound was as low as 1/6 of the time as compared to that with the fixed device. Tomato plants were irradiated with 40.5 kHz ultrasound for 2 weeks for 15 sec at 75-sec intervals for 2 weeks (I) or without irradiation (NI). Tomato wilt was suppressed the same as with continuous 2-week irradiation. The tomato wilt disease index was analyzed with R, Wilcoxon rank sum test. $p<0.05$, bars=standard error, Asterisk means significance as compared to NI.

2-week irradiation conferred a bigger effect (Fig. 2c).

The effect of ultrasound irradiation was observed in both cultivars, Moneymaker and Momotaro, which suggested that ultrasound irradiation is applicable to preventing wilt disease in any tomato cultivar.

2. Even with irradiation for 1/6 of the time, tomato wilt was suppressed

As the number of plants irradiated with the aerial ultrasound oscillator was limited because of the limited irradiation area (50 cm in diameter at a 70 cm distance from the oscillator), we developed a traveling device that irradiates 40.5 kHz ultrasonic (intermittent pulse wave; each plant was irradiated for 15 sec with a 75 sec interval, on average) to a larger number of seedling plants (Japanese patent 2016-26268 JP), and its disease control effect was tested. With the device, the total period for the irradiation of each plant by ultrasound diminished to 1/6 of the time as compared to with the fixed device. Wilt was suppressed in both cvs. Moneymaker and Momotaro (Fig. 3) with 2 weeks of ultrasound irradiation using the traveling device, as in the 2 weeks of irradiation using the fixed device.

The traveling 40.5 kHz aerial ultrasound oscillator device seems suited to producing a large number of tomato seedlings with resistance to wilt disease.

3. Ultrasound irradiation suppressed tomato powdery mildew and rice blast but did not suppress cabbage yellows

We examined whether the efficacy of ultrasound irradiation of 40.5 kHz in disease suppression depends on plant species or plant-pathogen interactions. For that purpose, we tested the disease suppression effect on tomato (cv. Momotaro) powdery mildew caused by *Oidium* sp., cabbage yellows caused by *Foc*, and rice (cvs. Aichi-asahi and Kinuhikari) blast caused by *Po*.

Powdery mildew and blast were significantly suppressed in ultrasound-irradiated tomato and rice plants as compared to non-irradiated plants (Fig. 4a, b). On the other hand, cabbage yellows was not suppressed by ultrasound irradiation (Fig. 4c). The irradiation of strawberry to 40.5 kHz ultrasound suppressed powdery mildew caused by *Sphaerotheca aphanis* (original data will be presented in another paper).[20] However, lettuce powdery mildew caused by *Erysiphe* sp. was not suppressed by 40.5 kHz ultrasound irradiation (Fig. S2).

These results suggested that disease suppression by ultrasound irradiation possibly differs depending on plant species or plant-pathogen interactions.

4. Ultrasound irradiation did not affect the growth of tomato and rice plants

Sound vibrations have been previously reported to promote or inhibit plant growth.[21–23] For example, tomato growth was promoted by audible sound (20–2000 Hz) irradiation.[22] However, no report on the effect of ultrasound on the growth of plants could be found. Furthermore, several reports have claimed that the induction of disease resistance in plants is sometimes accompanied by the inhibition of growth.[24,25] Therefore, we measured the heights of tomato (cv. Momotaro) and rice (cv. Aichi-

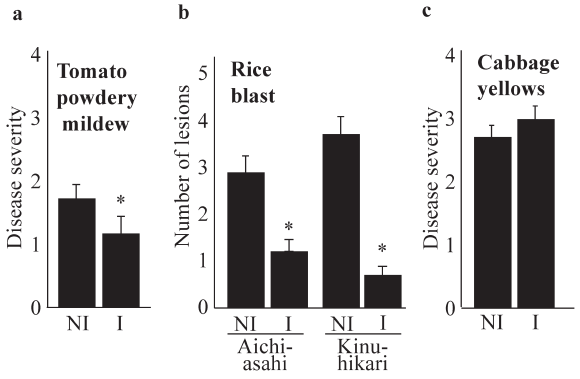

Fig. 4. Disease suppression by ultrasound irradiation possibly differs depending on the plant species or plant-pathogen interactions. One-week-old tomato seedlings (cv. Momotaro) were irradiated with 40.5 kHz ultrasound for two weeks and inoculated with tomato powdery mildew pathogens just after terminating ultrasound irradiation. Two weeks after inoculation, the disease incidence of each plant was graded from 0 to 4 using the following index: 0, no symptoms; 1, a foliar area of 0–5% indicating powdery mildew symptoms; 2, 6–30%; 3, 31–60%; 4, 61–100% (a). One-week-old rice seedlings (cvs. Aichi-asahi and Kinuhikari) were irradiated with ultrasound for two weeks and inoculated with rice blast pathogen (*Po*) just after terminating ultrasound irradiation. One week after inoculation, the lesions on the upper three leaves of each plant were counted (b). One-week-old cabbage seedlings were irradiated with ultrasound for two weeks and inoculated with cabbage yellows pathogens just after terminating ultrasound irradiation. Two weeks after inoculation, the disease incidence of each plant was graded from 0 to 4 using the following index: 0, no symptoms; 1, lower leaves yellowing; 2, lower and upper leaves yellowing; 3, lower leaves yellowing and wilting and upper leaves yellowing; 4, all leaves wilting and yellowing or dead (c). The disease indexes of tomato wilt and cabbage yellows were analyzed with R, Wilcoxon rank sum test, and the number of lesions of rice blasts was analyzed with R, *t*-test. $p<0.05$, bars=standard error, Asterisk means significance as compared to NI.

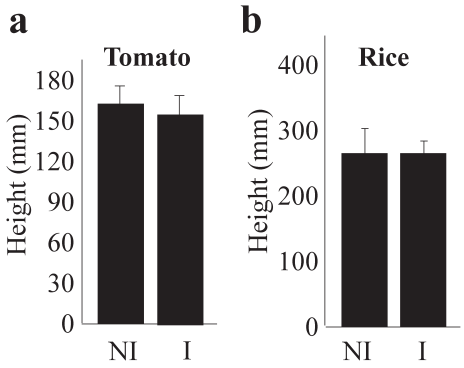

Fig. 5. The heights of tomato and rice plants did not change with ultrasound wave irradiation. We examined the effects of ultrasound on plant growth. The heights of tomato (cv. Momotaro) and rice (cv. Aichiasahi) plants did not show differences as compared to NI plants. The heights of tomato and rice plants were analyzed with R, *t*-test. $p<0.05$, bars=standard error.

asahi) plants after 2 weeks of irradiation at 40.5 kHz ultrasound. Ultrasound conditions were identical to those described previously. No significant positive or negative effect was observed on the growth of tomato and rice plants (Fig. 5).

5. The SA signaling pathway was associated with the suppression of tomato wilt

As ultrasound seemed to induce disease resistance in tomatoes, we tried to profile the expression of resistance-related genes in ultrasound-irradiated tomatoes (cv. Moneymaker) by RT-qPCR. Target genes *PAL1* (M83314 in GenBank), *NIM1* (also known as *NPR1*; AY640378), and *PR1a* (M69247) are involved in the SA signaling pathway, and *LoxD* (U37840) is involved in the jasmonic acid (JA) signaling pathway (Fig. S1).[26–29] Choi *et al.* (2017) reported that sound irradiation (1000 Hz, 3 hr/day, 10 days) primed SAR in *A. thaliana*, and the expression of SA signaling pathway-related genes in sound-irradiated plants was upregulated earlier than in non-irradiated plants when inoculated with *B. cinerea*.[9]

Thus, in this study, we extracted total RNA from the leaves, stems, and roots of 40.5 kHz ultrasound-irradiated or non-irradiated tomato plants at 0 and 24 hpi with *Fol*. As a control, we used tomato plant foliage sprayed with 100 ppm validamycin A (VMA, Sumitomo Chemical Co.) 1 week before inoculation. VMA is known to induce SAR in tomatoes and suppress Fusarium wilt.[24,25]

The expression of *PR1a* in leaves was significantly upregulated by ultrasound irradiation at 0 hpi, although it was lower than with VMA treatment (Fig. 6). The expression of *LoxD* in leaves and stems was downregulated at 0 hpi. The expression of *PAL1* or *NIM1* was not significantly upregulated, although the expression of both genes was upregulated by VMA in tomato leaves.

No gene in ultrasound-irradiated tomatoes showed higher expression than in non-irradiated plants at 24 hpi, while VMA treatment significantly induced the expression of all genes (*PAL1*, *NIM1*, and *PR1a*) involved in SAR. Especially the expression of *NIM1* was downregulated by ultrasound irradiation at 24 hpi.

These results showed that ultrasound irradiation possibly induced a part of the SA signaling pathway and reduced Fusarium wilt in tomato plants, which did not contradict previous reports by Ishikawa *et al.* (2005),[24] Di *et al.* (2016),[30] and Choi *et al.* (2017).[9] In our study, no gene involved in the SA signaling pathway was upregulated at 24 hpi in ultrasound-irradiated tomato plants (Fig. 6), which did not contradict a previous report where *PR1* (the homolog of *PR1a* in tomato) in *A. thaliana* was downregulated when infected with *F. oxysporum* at 1 day post inoculation (dpi).[31]

In conclusion, we found that ultrasound irradiation induced

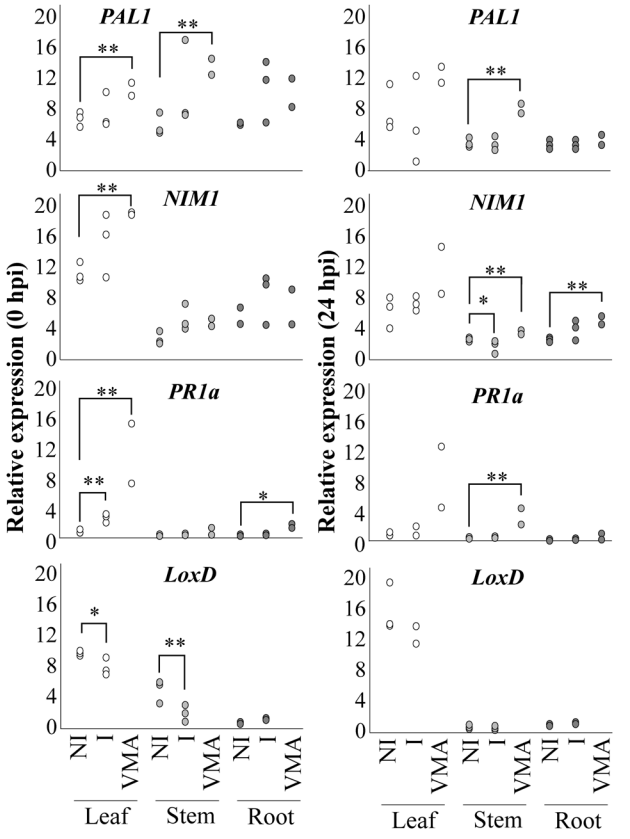

Fig. 6. The expression of *PR1a* possibly involved in the SA signaling pathway was upregulated in tomato plants irradiated by ultrasound. Analyses of the expression of defense-related genes were performed by RT-qPCR. Genes suggested to be involved in the SA signaling pathway—*PAL1*, *NIM1*, and *PR1a*—and a gene involved in the JA-signaling pathway—*LoxD*—were examined. *PR1a* was upregulated in leaves at 0 hpi, and *LoxD* was downregulated in leaves and stems at 0 hpi. Total RNA was isolated from leaves (white circle), stems (light gray), and roots (dark gray) of tomato plants (cv. Moneymaker) irradiated with ultrasound (40.5 kHz, *ca.* 100 dB, intermittent pulse wave, 2 weeks) at 0 and 24 hr post inoculation (hpi). The relative expression levels of mRNA were determined by normalizing the PCR threshold cycle number of each gene with that of the *Actin2* reference gene. Three biological replicates were used in the experiments. The relative expression levels of the mRNA of I were compared to those of NI and analyzed with R, *t*-test. *, $p<0.10$; **, $p<0.05$, bars=standard error.

disease resistance that lasted longer than a week in tomato and rice plants. Ultrasound irradiation can provide seedlings with resistance to later attacks by pathogens.

Preliminary studies on the effect by irradiation at lower frequencies indicated that tomato wilt is suppressed in plants irradiated with not only 40.5 but also 19.8 and 28.9 kHz. Although the difference was small, 19.8 kHz showed a higher preventive effect than did 40.5 kHz, which indicated that the efficacy of the prevention effect differs depending on the frequencies of ultrasound. In the future, we are planning to optimize the wavelength of ultrasonic waves to be irradiated.

Ultrasound is easily absorbed by PVC. Therefore, in the practical use of ultrasound in a greenhouse, ultrasound can be easily shielded to prevent unexpected negative effects on humans or the environment. In the experiment using strawberries in a greenhouse, no effect was observed on the ratio of pollination by bees or the number of spider mites (personal communication from M. Arimoto and T. Nishimura).

The power consumption of the ultrasound oscillator used in our experiment is 40 W, which is the same as that of a room lamp; it can expose more than 200 plant pots (7.5 cm in diameter) under the traveling device (3 m×50 cm in the irradiation area), for example. The ultrasound device seems to be applicable in ecofriendly agricultural fields.

Further studies are required to determine more effective and efficient conditions for ultrasound irradiation, how long the induction of disease resistance lasts, and how plants recognize ultrasound stimuli. Each plant species might have a unique sensor or recognition mechanism because the efficacy of disease suppression depends on the plant species (Fig. 4). Moreover, it is necessary to examine which plants (or diseases) will respond to the application of ultrasound. In this study, we found that aerial ultrasound irradiation is effective for suppressing not only tomato powdery mildew caused by airborne *Oidium* sp. but also tomato wilt caused by soilborne *Fol*, perhaps by inducing *PR1a* involved in the SA signaling pathway in plants. This is consistent with previous reports that described the suppression of rice blast and tomato wilt by SAR inducers, such as PBZ and VMA.[24,32] All of the pathogens that were controlled by ultrasound irradiation in this study are recognized as biotrophic. In general, plant resistance to biotrophic pathogens is mainly regulated by SA-mediated resistance, and resistance to necrotrophic pathogens is regulated by JA-mediated resistance.[26] However, it is known that even parts of necrotrophic fungi have a biotrophic stage during their infections, and they are suppressed by the induction of SA-mediated resistance.[33] It is expected that ultrasound irradiation suppresses diseases caused by pathogens that have a biotrophic stage during infection.

Acknowledgements

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References

1) A. Sham, K. Moustafa, S. Al-Ameri, A. Al-Azzawi, R. Iratni and S. AbuQamar: *PLoS One* **10**, e0125666 (2015).
2) E. W. Chehab, E. Eich and J. Braam: *J. Exp. Bot.* **60**, 43–56 (2008).
3) T. Arie and H. Nakashita: "Plant Activator," Plant Protection, Tokyo, pp. 531–536 (2007) (in Japanese).
4) P. P. Reddy: "Plant Defense Activators," Springer India, 2013.
5) K. Creath and G. E. Schwartz: *J. Altern. Complement. Med.* **10**, 113–122 (2004).
6) Y. C. Qin, W. C. Lee, Y. C. Choi and T.-W. Kim: *Ultrasonics* **41**, 407–411 (2003).
7) M. Gagliano, S. Mancuso and D. Robert: *Trends Plant Sci.* **17**, 323–325 (2012).
8) W. Bochu, S. Jiping, L. Biao, L. Jie and D. Chuanren: *Colloids Surf. B Biointerfaces* **37**, 107–112 (2004).
9) B. Choi, R. Ghosh, M. A. Gururani, G. Shanmugam, J. Jeon, J. Kim, S.-C. Park, M. J. Jeong, K. H. Han, D. W. Bae and H. Bae: *Sci. Rep.* **7**, 2527 (2017).
10) S. R. G. Beckett, S. Aspley, M. Graham and C. A. Marsden: *Psychopharmacology (Berl.)* **127**, 384–390 (1996).
11) A. Kershenbaum, D. T. Blumstein, M. A. Roch, Ç. Akçay, G. Backus, M. A. Bee, K. Bohn, Y. Cao, G. Carter, C. Cäsar, M. Coen, S. L. DeRuiter, L. Doyle, S. Edelman, R. Ferrer-i-Cancho, T. M. Freeberg, E. C. Garland, M. Gustison, H. E. Harley, C. Huetz, M. Hughes, J. Hyland Bruno, A. Ilany, D. Z. Jin, M. Johnson, C. Ju, J. Karnowski, B. Lohr, M. B. Manser, B. McCowan, E. Mercado 3rd, P. M. Narins, A. Piel, M. Rice, R. Salmi, K. Sasahara, L. Sayigh, Y. Shiu, C. Taylor, E. E. Vallejo, S. Waller and V. Zamora-Gutierrez: *Biol. Rev. Camb. Philos. Soc.* **91**, 13–52 (2016).
12) R. Mankin: *Perspect. Agric. Vet. Sci. Nutr. Nat. Resour.* **7**, 001, DOI: 10.1079PAVSNNR20127001 (2012).
13) A. Morozova, E. Zubkov, T. Strekalova, Z. Kekelidze, Z. Storozeva, C. Schroeter, N. A. Bazhenova, K.-P. Lesch, B. Cline and V. H. Chekhonin: *Prog. Neuropsychopharmacol. Biol. Psychiatry* **68**, 52–63 (2016).
14) K. Inami, T. Kashiwa, M. Kawabe, A. Onokubo-Okabe, N. Ishikawa, E. R. Pérez, T. Hozumi, L. A. Caballero, F. C. de Baldarrago, M. J. Roco, K. A. Madadi, T. L. Peever, T. Teraoka, M. Kodama and T. Arie: *Microbes Environ.* **29**, 200–210 (2014).
15) T. Kashiwa, K. Inami, M. Fujinaga, H. Ogiso, T. Yoshida, T. Teraoka and T. Arie: *J. Gen. Plant Pathol.* **79**, 412–421 (2013).
16) S. Urayama: *J. Gen. Plant Pathol.* **81**, 97–102 (2015).
17) Y. Kanda: *Bone Marrow Transplant.* **48**, 452–458 (2012).
18) S. R. Uppalapati, P. Ayoubi, H. Weng, D. A. Palmer, R. E. Mitchell, W. Jones and C. L. Bender: *Plant J.* **42**, 201–217 (2005).
19) M. Du, J. Zhao, D. T. W. Tzeng, Y. Liu, L. Deng, T. Yang, Q. Zhai, F. Wu, Z. Huang, M. Zhou, Q. Wang, Q. Chen, S. Zhong, C. Li and C. Li: *Plant Cell* **29**, 1883–1906 (2017).
20) M. Arimoto, Y. Shimokawa, M. Yamamoto, Y. Enami, H. Amano, T. Iwagaya, A. Goto, T. Yoshida, D. Kawakami and T. Arie: *Jpn. J. Phytopathol.* **83**, 196 (2017) (in Japanese).
21) R. H. E. Hassanien, T. Hou, Y. Li and B. Li: *J. Integr. Agric.* **13**, 335–348 (2014).
22) T. Z. Hou and R. E. Mooneyham: *Am. J. Chin. Med.* **27**, 1–10 (1999).
23) H. Hunan Sheng nong ye ke xue yuan and J. ShiRen : *Agric. Sci. Technol.* **12**, 847–851 (2011).
24) R. Ishikawa, K. Shirouzu, H. Nakashita, H.-Y. Lee, T. Motoyama, I. Yamaguchi, T. Teraoka and T. Arie: *Phytopathology* **95**, 1209–1216 (2005).

25) R. Ishikawa, K. Shirouzu, H. Nakashita, T. Teraoka and T. Arie: *J. Pestic. Sci.* **32**, 83–88 (2007).
26) J. Glazebrook: *Annu. Rev. Phytopathol.* **43**, 205–227 (2005).
27) S. Hase, S. Takahashi, S. Takenaka, K. Nakaho, T. Arie, S. Seo, Y. Ohashi and H. Takahashi: *Plant Pathol.* **57**, 870–876 (2008).
28) J. A. Ryals, U. H. Neuenschwander, M. G. Willits, A. Molina, H. Y. Steiner and M. D. Hunt: *Plant Cell* **8**, 1809–1819 (1996).
29) Y. Bai, S. Sunarti, C. Kissoudis, R. G. F. Visser and C. G. van der Linden: *Front. Plant Sci.* **9**, 801 (2018).
30) X. Di, F. L. W. Takken and N. Tintor: *Front. Plant Sci.* **7**, 170 (2016).
31) B. N. Kidd, N. Y. Kadoo, B. Dombrecht, M. Tekeoğlu, D. M. Gardiner, L. F. Thatcher, E. A. B. Aitken, P. M. Schenk, J. M. Manners and K. Kazan: *Mol. Plant Microbe Interact.* **24**, 733–748 (2011).
32) K. Yoshioka, H. Nakashita, D. F. Klessig and I. Yamaguchi: *Plant J.* **25**, 149–157 (2008).
33) Y. Kouzai, M. Kimura, M. Watanabe, K. Kusunoki, D. Osaka, T. Suzuki, H. Matsui, M. Yamamoto, Y. Ichinose, K. Toyoda, T. Matsuura, I. C. Mori, T. Hirayama, E. Minami, Y. Nishizawa, K. Inoue, Y. Onda, K. Mochida and Y. Noutoshi: *New Phytol.* **217**, 771–783 (2018).
34) Y. Kanemaru, T. Yoshida, T. Mizukami, N. Tanaka, T. Teraoka and T. Arie: *Jpn. J. Phytopathol.* **79**, 73 (2013) (in Japanese).
35) D. Kawakami, T. Yoshida, T. Mizukami, H. Amano, T. Iwagaya, H. Aritake, A. Goto, Y. Enami, M. Yamamoto, M. Arimoto, T. Teraoka, K. Komatsu, T. Fukuhara and T. Arie: *Jpn. J. Phytopathol.* **83**, 199 (2017) (in Japanese).