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Reactive short-chain leaf volatiles act as powerful inducers of abiotic stress-related gene expression

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Abiotic stresses cause serious damage to plants; therefore, plants undergo a complicated stress response through signal transduction originating from environmental stimuli. Here we show that a subset of short-chain leaf volatiles with an α , β -unsaturated carbonyl bond in their structure (reactive short-chain leaf volatiles, RSLVs) like (*E*)-2-hexenal and (*E*)-2-butenal can act as signal chemicals that strongly induce the gene expression of abiotic-related transcription factors, such as heat stress-related transcription factors (*HSA2*, *MBF1c*) and other abiotic stress-related transcription factors (*DREB2A*, *ZATs*). RSLV-induced expression of *HSA2* and *MBF1c* was eliminated in *HSA1s*-, known as heat stress response master regulators, knockout mutant, whereas those of *DREB2A* and *ZATs* were not, suggesting that the RSLV signaling pathway is composed of *HSA1*-dependent and -independent pathways. RSLV treatment induced production of chaperon proteins, and the RSLV-treated *Arabidopsis* thus demonstrated enhanced abiotic stress tolerance. Because oxidative stress treatment enhanced RSLV production, we concluded that commonly found RSLVs produced by environmental stresses are powerful inducer of abiotic stress-related gene expression as oxidative stress signals.

As sessile organisms, plants are constantly exposed to widely varying and unfavorable environmental conditions, such as drought and extreme temperature, which are major limiting factors in crop production¹. Under environmental stress, disturbance of the metabolic balance in oxidative organelles often results in enhanced production of reactive oxygen species (ROS)². The chloroplast in stressed conditions is one of the major sites producing ROS; disturbance of the photochemical reaction leads to ROS production, which is enhanced by conditions limiting CO₂ fixation such as drought and salt stresses and the combination of these conditions with high light^{2,3}. Plants experiencing environmental stresses exhibit complicated responses to overcome the stresses. In the response process, physical stimuli from the environment are perceived and transduced to biochemical processes, resulting in the induction of a series of abiotic stress-related gene expression. Recently, ROS itself⁴, ROS-related chemicals such as carotenoid oxidation products⁵ and lipophilic reactive electrophilic species⁶ are recognized as important signaling chemicals involved in environmental stress responses in addition to phytohormones such as abscisic acid, salicylic acid, jasmonic acid and ethylene.

Previously, we determined that short-chain α , β -unsaturated carbonyls are generated from peroxidized polyunsaturated fatty acids (PUFAs) such as linolenic acid (C18:3) and *cis*-3, *cis*-9, *cis*-12-hexadecatrienoic acid (C16:3), the richest PUFAs in thylakoid membranes⁷. Through their highly reactive electrophilic α , β -unsaturated carbonyl bonds, these carbonyls can easily modify proteins in stressed plants^{7,8}. In this study, we name such short-chain volatiles having α , β -unsaturated carbonyl bonds in their structure reactive short-chain leaf volatiles (RSLVs) and hypothesize that RSLVs act as signal molecules to induce abiotic gene expression because chemicals with α , β -unsaturated carbonyl bonds have been pointed out to be biologically active^{9,10}.

Results and discussion

RSLVs induced various abiotic-related transcription factors. We used (*E*)-2-hexenal as a model RSLV because (*E*)-2-hexenal is a widely distributed C6 RSLV known as a green leaf volatile (GLV). An overview of the whole gene expression pattern obtained by a comprehensive microarray analysis showed that vaporized (*E*)-2-hexenal treatment (10 nmol cm⁻³ for 30 min, photograph is shown in Supplementary Fig. S1a) induced genes up-regulated in various abiotic stress responses (Fig. 1a, lane RSLV1). Comparing gene expression patterns under various abiotic stresses allowed us to classify up-regulated genes into the following 5 groups (Fig. 1a): heat, UV-B and oxidative stresses-responsive genes (Group A); various stresses-responsive genes (Group B); genes that



respond to salt, oxidative, osmotic drought, cold and wounding (Group C); salt- and osmotic-responsive genes (Group D); and cold-responsive genes (Group E). Among these groups, gene expression of Groups A and B were stimulated by (*E*)-2-hexenal treatment. Many of the 100 most highly up-regulated genes were abiotic stress-related genes (Fig. 1b, Supplementary Table S1) as follows: genes encoding HSPs (21 genes); heat or oxidative stress-related transcription factors (7 genes), including *HSFA2*¹¹, *MBF1c*¹² and *ZATs*¹³; abiotic-related AP2/ERF transcription factors (10 genes), including *DREB2A*¹⁴; and other transcription factors (5 genes).

RSLVs strongly and rapidly induced *HSFA2* gene expression. Because *HSFA2* and *HSPs* were prominently induced by (*E*)-2-hexenal treatment, detailed biological activity of (*E*)-2-hexenal was examined using the heat shock factor (HSF)-heat shock protein (HSP) system. Vaporized (*E*)-2-hexenal rapidly and powerfully induced *HSFA2* expression within 30 min (Fig. 1c). This expression was transient and terminated after 2 h. We confirmed that these treatments increased the internal (*E*)-2-hexenal concentration comparable to the intracellular concentration of intact higher plants^{15,16}. At 10 min after applying (*E*)-2-hexenal at 10 nmol cm⁻³ to *Arabidopsis*, the internal concentration of (*E*)-2-hexenal exhibited a transient increase of up to 25 nmol g⁻¹ fresh weight (FW) (supplementary Fig. S1b, c). We tested the effects of other GLVs on *HSFA2* expression: (*E*)-2-hexenal, (*Z*)-3-hexenal, and *n*-hexenal did not induce *HSFA2* expression (Fig. 1d). Among ketones, (*E*)-3-hepten-2-one but not 2-heptanone induced *HSFA2* expression, suggesting that the α , β -unsaturated carbonyl bond moiety was essential for *HSFA2* induction. Induction of *HSFA2* was dose-dependent above 2.5 nmol cm⁻³ and saturated at 25 nmol cm⁻³ (Supplementary Fig. 2).

RSLVs having longer hydrocarbon chains act as signal molecules with less cytotoxic effect. To determine the effect of carbon chain length on *HSFA2* induction, we compared the effects of a series of RSLVs with various carbon chain lengths, and found that RSLVs with chain lengths of C4 to C9 were effective with a slightly higher induction in the aldehyde form than in the ketone form (Fig. 2). Expression patterns obtained by microarray analyses using (*E*)-2-butenal- or (*E*)-3-hepten-2-one-treated *Arabidopsis*, were essentially homologous to that of (*E*)-2-hexenal (Fig. 1a, lane RSLV2 and 3), suggesting that the RSLVs exhibit identical induction activity against abiotic stress-related genes. However, a member of RSLVs is potentially photosynthesis damaging agents, as shown by our previous *in vitro* study⁸. The ratio F_v/F_m , the maximum photochemical quantum efficiency of photosystem II (PSII), is used as a measure of stress response because PSII is one of the sites most sensitive to α , β -unsaturated carbonyls^{8,10}, thus we examined the photosynthesis damaging activity of RSLVs by measuring F_v/F_m in *Arabidopsis* at 23°C. As a result, except vinyl group (H₂C=CH-) containing RSLVs such as 2-propenal, 1-buten-3-one, and 1-penten-3-one, RSLVs did not damage PSII (Fig. 2, lower panel, Supplementary Fig. S3). Therefore, the RSLVs with chain lengths of C4 to C9 having no vinyl group possibly act as signal molecules with less cytotoxic effect.

Evaluation of endogenous RSLVs on *HSFA2* expression. Production of (*E*)-2-hexenal via an enzymatic pathway (Supplementary Fig. S4a) is assumed to be activated when plant tissues are disintegrated by physical factors, such as pest invasion, wounding or freezing¹⁷. In *Arabidopsis* ecotype No-0, which can produce (*E*)-2-hexenal enzymatically, the (*E*)-2-hexenal content in leaves reached 110 nmol g⁻¹ FW when the leaf was disrupted¹⁶. However, in *Arabidopsis* ecotype Col-0 used in this study, (*E*)-2-hexenal is undetectable because hydroperoxide lyase, which is necessary for producing C6 GLVs, is nonfunctional truncated protein due to 10-nucleotide deletion in its

first exon¹⁸. This difference allowed us to determine which RSLVs were produced by non-enzymatic peroxidation of PUFAs. Oxidative stress caused by treatment with 10 μ M methylviologen (MV) under illumination resulted in an increase of (*E*)-2-butenal (Supplementary Fig. S5a). Full-scan spectra of fragment ions (Supplementary Fig. S5b) and selected reaction monitoring using major fragment ions (Supplementary Fig. S5c) confirmed the identity of this endogenous compound. Concomitant with increase in *HSFA2* mRNA expression (Supplementary Fig. S6a, column Col-0), these results suggest that (*E*)-2-butenal produced by non-enzymatic peroxidation of PUFAs (possible pathway is shown in Supplementary Fig. S4b) can act as signaling chemicals that induce *HSFA2* expression.

To assess the effect of endogenous RSLVs on *HSFA2* expression, we analyzed *HSFA2* mRNA expression in *aor* mutants, which are deficient in chloroplastic alkenal/one oxidoreductase (AOR)¹⁹, the enzyme that catalyzes the saturation of α , β -unsaturated carbonyl bonds in reactive carbonyls such as RSLVs (Supplementary Fig. S6e). Previously, we found that *aor* exhibited high sensitivity to MV treatment concomitant with accumulation of reactive carbonyls including RSLVs²⁰. In this study, both *aor* and Col-0 showed similar levels of (*E*)-2-butenal and *HSFA2* expression under normal condition, and MV treatment enhanced accumulation of (*E*)-2-butenal and *HSFA2* expression in *aor* and Col-0 (Supplementary Fig. S6a, b). The enhancement of *HSFA2* expression in the MV-treated *aor* was higher than that in MV-treated Col-0, corresponding to higher (*E*)-2-butenal accumulation in *aor* (Supplementary Fig. S6a, b). This result indicates that total of RSLVs including (*E*)-2-butenal and minor RSLVs whose concentration is lower than detection-limit of our analysis might induce *HSFA2* higher in *aor* mutant than Col-0. Similar results were obtained from ultraviolet B (UV-B) stress treatment (Supplementary Fig. S6c, d) that accompanies ROS production²¹ and lipid peroxidation²². These enhancements of *HSFA2* expression by stress treatments support the hypothesis that RSLVs are involved in the induction of *HSFA2* expression *in vivo*.

RSLV signaling is transmitted via both HSF1-dependent and -independent pathways. In a heat-stress response, HSF1s act as master regulators to drive the HSF-HSP system²³. To determine whether RSLV stimulates HSF1-mediated *HSP* expression, we used an *HSFA1a/1b/1d/1e* quadruple knockout mutant (*QK*), which exhibited no *HSFA2* expression when exposed to heat conditions²³. In the genomic background ecotypes of *QK*, wild-type Col-0 and *Ws-0*, *HSFA2* and *MBF1c* (belonging to group A, Fig. 3a), and *DREB2A*, *ZAT10* and *ZAT12* (belonging to group B, Fig. 3a) were induced by (*E*)-2-hexenal treatment (Fig. 3b, c). In (*E*)-2-hexenal-treated *QK*, *HSFA2* and *MBF1c* expression was almost eliminated (Fig. 3b), whereas expression of *DREB2A* and *ZAT10* remained at a similar level to that of *Ws-0* (Fig. 3c). These results suggested that RSLV-mediated gene expression involves heterogeneous pathways, i.e. the HSF1-dependent and HSF1-independent pathways.

Tomato *HSFA2s*, and rice *HSFA2s* and *DREB2A* were also induced by (*E*)-2-hexenal. (*E*)-2-Hexenal is a common RSLV that is also detected in tomato²⁴ and rice²⁵, thus might act as a signal chemical among various plant species. To explore this possibility, we examined the induction of HSFs by (*E*)-2-hexenal in a model tomato cultivar (*Solanum lycopersicum* 'Micro-Tom') and rice (*Oryza sativa* 'Nipponbare') because these species in which the heat-stress response mechanism has been well-characterized. The results of tomato were similar to those described for *Arabidopsis* in that the tomato *HSFA2* genes were induced by (*E*)-2-hexenal. The induction profiles of other classes of HSFs—*HSFA1* and *HSFB1* in tomato were similar to those in *Arabidopsis* (Supplementary Fig. S7a, b). In the case of a monocotyledonous plant rice, (*E*)-2-hexenal also up-regulated heat-inducible *OsHSFA2s* and abiotic stress-inducible *OsDREB2A* (Supplementary Fig. S7c). These similarities of gene

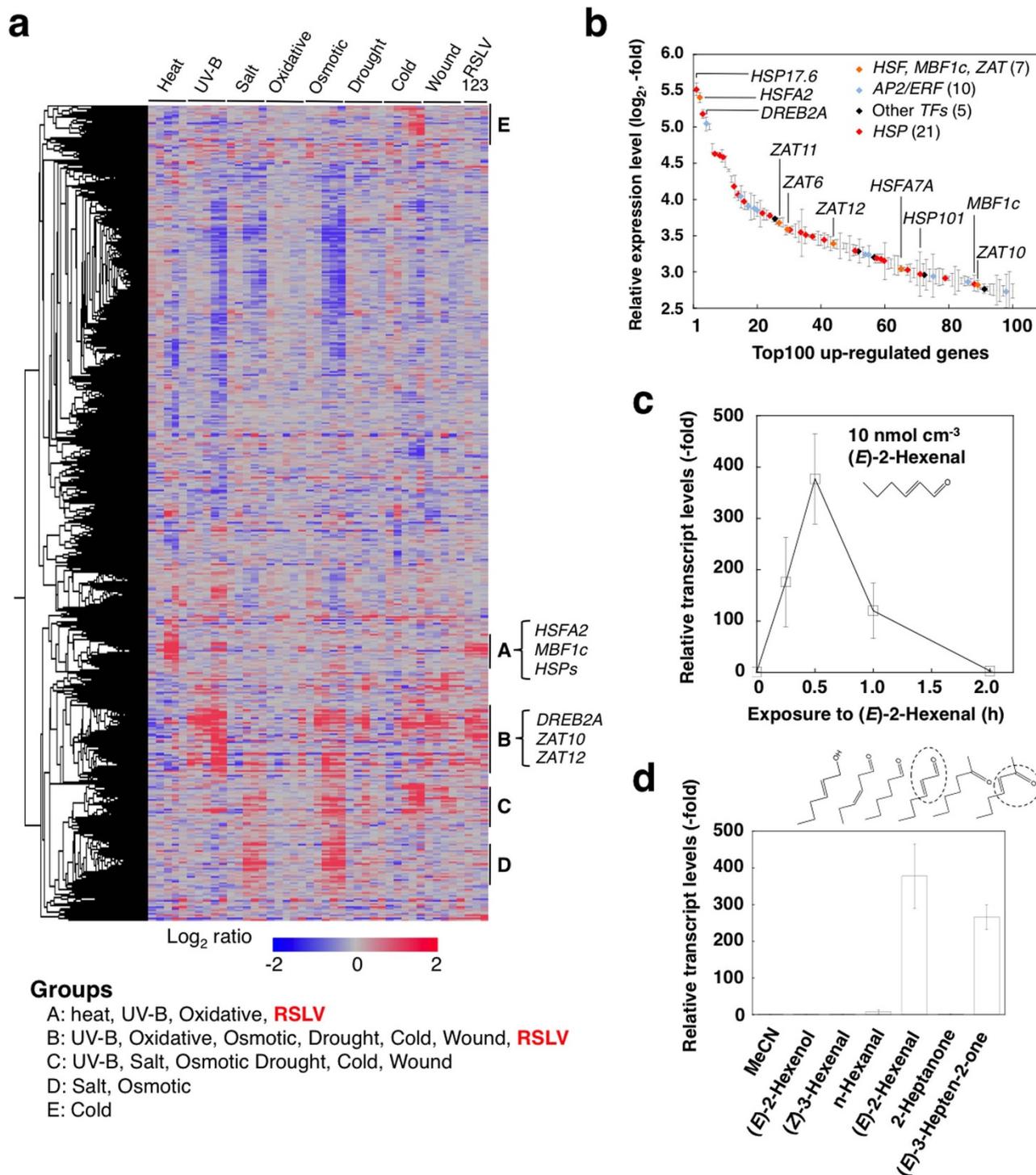


Figure 1 | Abiotic stress-related genes were up-regulated by RSLV treatment in *Arabidopsis*. (a), Heat map constructed with the whole gene expression. Expression data used are data from shoots collected over the course (0.25, 0.5, 1, 3, 6 h of heat, UV-B, drought or wound stresses) and (0.5, 1, 3, 6, 12 h of oxidative, salt, osmotic or cold stresses) obtained from the AtGenExpress database. RSLVs used for obtaining expression data are (*E*)-2-hexenal (lane 1), (*E*)-2-butenal (lane 2) and 3-hepten-2-one (lane 3). Genes induced by heat and various stresses induced were classified into Groups A and B, respectively. In addition, responsive genes to salt, oxidative, osmotic drought, cold and wound (Group C), salt- and osmotic-responsive genes (Group D) and cold-responsive genes (Group E) are shown. (b), Transcription factors and HSPs in the 100 most highly up-regulated genes. Expression was induced by (*E*)-2-hexenal treatment. Symbols indicate the *HSF* and *ZAT* genes (orange), *AP2/ERF* genes (blue), other transcription factor genes (black), and *HSP* genes (red). Symbols of other genes are omitted. Data are means \pm SE ($n = 3$). Detailed list is shown in Table S1. (c) and (d), *Arabidopsis* plants were exposed to (*E*)-2-hexenal (10 nmol cm^{-3}) for various time periods (0 to 2 h) (c), or series of C6 GLVs and their analogues (each 10 nmol cm^{-3}) for 30 min (d).

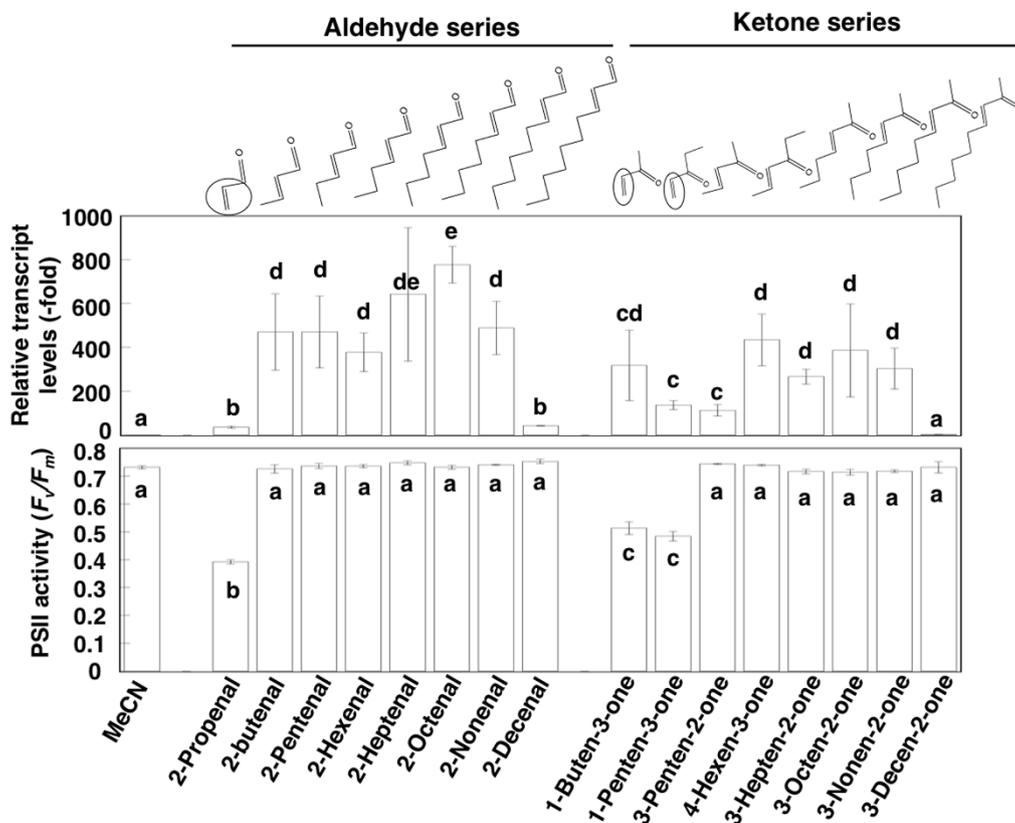


Figure 2 | Effect of chain-length of RSLVs on Induction of *HSFA2* expression and PSII activity. *Arabidopsis* plants were exposed to (*E*)-2-hexenal (10 nmol cm^{-3}) a series of RSLVs of aldehyde form (each 10 nmol cm^{-3}) for 30 min, or a series of RSLVs of ketone form (each 10 nmol cm^{-3}) for 30 min (upper panel). Expression of the *HSFA2* gene was determined using qRT-PCR. Relative transcript levels were normalized to *ACTIN2* mRNA. The expression level of the 0 h exposure sample was set to 1. Effect of RSLVs on PSII activity was determined by treatment of the indicated α , β -unsaturated aldehydes or ketones (each 25 nmol cm^{-3}) for 90 min to *Arabidopsis* plants, and then PSII activity was measured (lower panel). The chemical structures of the RSLVs are shown above the graphs. Dotted and solid circles indicate the α , β -unsaturated carbonyl bond moieties and vinyl group, respectively. Data are means \pm SE ($n = 3$ in upper panel, $n = 5$ in lower panel). Values followed by the same letter are not significantly different according to Tukey-Kramer ($P < 0.05$).

expression profiles among species indicate that RSLVs might be common signal chemicals.

RSLV treatment could enhance abiotic stress tolerance. Because RSLVs induced *HSF* and *HSP* gene expression, protein expression enhancement by RSLV treatment was confirmed by detecting two HSPs: HSP101 (encoded by At1g74310) and HSP17.6 (encoded by At1g53540). As shown in Fig. 4a, HSP101 and HSP17.6 were induced by (*E*)-2-hexenal, (*E*)-2-butenal and (*E*)-3-hepten-2-one treatment at 23°C within 2 h, whereas the levels in acetonitrile (MeCN)-treated control plants remained low. Finally, we investigated an effect of RSLV treatment on abiotic stress tolerance. RSLV-induced thermotolerance was assessed by evaluating hypocotyl elongation²⁶ and survival tests, because RSLV treatment could enhance *HSFA2* expression and HSP17.6 production (Supplementary Fig. S8) those confer acquired thermotolerance²⁷. After 2.5 days of growth on vertical plates in the dark, the seedlings were applied to the hypocotyl elongation test (Fig. 4b). After heat treatment at 45°C for 2 h, the control and solvent-control seedlings stopped developing, whereas seedlings pretreated at 38°C for 90 min (for gaining acquired thermotolerance) or RSLV-treated seedlings at 23°C for 120 min continued hypocotyl elongation. The thermotolerance enhancing effect of (*E*)-2-hexenal was not observed in *HSFA1*-deficient *QK* mutants (Supplementary Fig. S9a), indicating that the physiological importance of *HSFA1*-dependent pathway in heat stress response. In the survival enhancement test, RSLV treatments enhanced thermotolerance similar level to that of acquired thermotolerance (Fig. 4c). In addition, RSLV treatments could enhance protection

of PSII from heat- or UV-B-derived damages (Supplementary Fig. S9b, c).

Possible role of RSLVs in abiotic stress responses. RSLVs have been widely detected among plant species, and increased RSLV production has been observed under abiotic stresses⁶ including heat stress (Fig. S10). Production of (*E*)-2-hexenal was detected in a photoinhibition-sensitive *Arabidopsis* mutant (Col-0 background) *npq1* by intense light conditions²⁸, which could cause ROS to be overproduced from the loss of energy dissipation. Additionally, in tomato plants, production of (*E*)-2-hexenal was enhanced under heat and cold stresses²⁹. Furthermore, increased levels of (*E*)-2-pentenal and (*E*)-2-hexenal were also detected in tobacco plants under photooxidative stress condition¹⁵. Consequently, non-enzymatic pathway-derived small 2-alkenals, and both enzymatic and non-enzymatic pathway-derived (*E*)-2-hexenal can act as endogenous signal chemicals that respond to abiotic stresses.

Volatiles such as isoprenoids play important roles in various stresses tolerance³⁰. Also in the case of RSLVs, our results indicate that RSLVs stimulate heterogeneous signal transduction in response to abiotic stress (Fig. 5). One signal transduction pathway is an *HSFA1*-dependent pathway expressing proteotoxic stress-related genes that contribute to HSP production to maintain protein homeostasis. The other signal transduction pathway is mediated by *HSFA1*-independent pathway expressing various abiotic stress-response genes. RSLVs stimulate both pathways as oxidative stress signals to induce proteotoxic and abiotic stress-response genes.

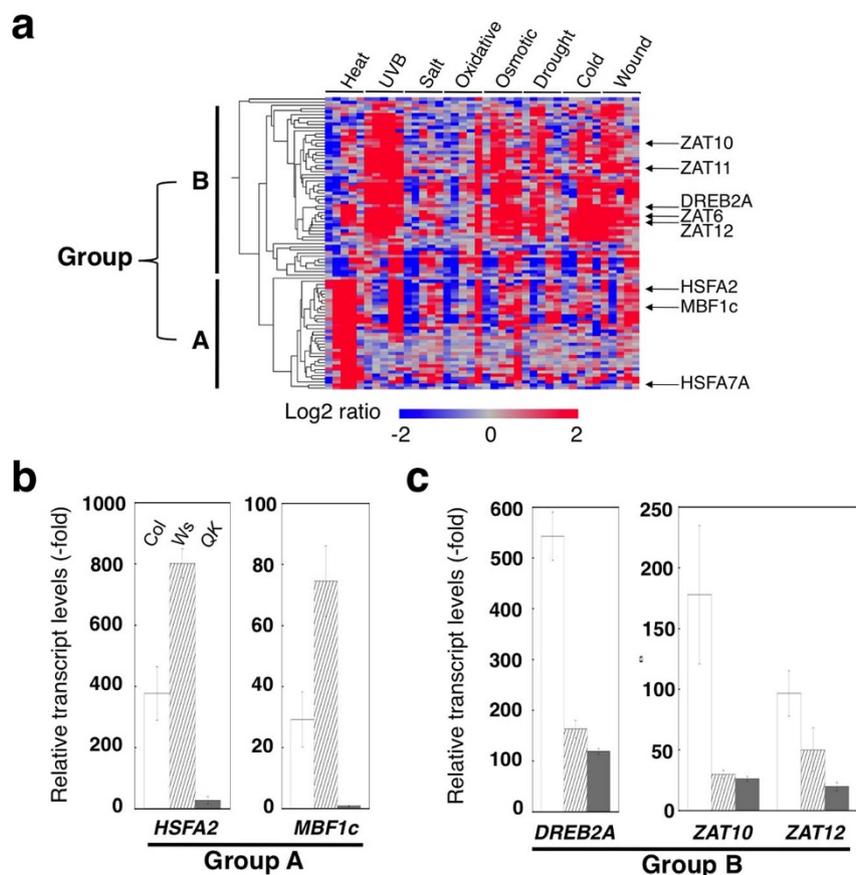


Figure 3 | RSLV-induced genes are divided into HSF1-dependent or -independent groups. (a), Heat map constructed with the 100 most highly up-regulated genes by (*E*)-2-hexenal treatment. (b), Expression of transcription factors in (*E*)-2-hexenal-treated Col-0 (Col, open column), Ws-0 (Ws, shaded column) and HSF1A quadruple knockout mutant (QK, gray column). A and B mean groups classified in panel (a).

Methods

Chemicals. 2-Propenal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*)-2-nonenal, (*E*)-2-decenal, (*E*)-3-hepten-2-one, (*E*)-3-octen-2-one, (*E*)-3-nonen-2-one, (*E*)-3-decen-2-one, (*E*)-2-hexenol, 2-hexanal, and 2-heptanone were purchased from Tokyo Chemical Industry (Tokyo, Japan). 1-Penten-3-one and 3-penten-2-one were purchased from Sigma-Aldrich (St. Louis, MO, USA). (*Z*)-3-Hexenal was obtained from Bedoukian Research Inc. (Danbury, CT, USA). (*E*)-2-Hexenal, (*E*, *Z*)-4-hexen-3-one, and other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

Plant materials. Seeds of *Arabidopsis thaliana* (ecotype Columbia-0: Col-0; Wassilewskija: Ws-0) and tomato (*Solanum lycopersicum* ‘Micro-Tom’) were sown on Jiffy-7 peat pellets (Sakata Seed Co., Yokohama, Japan) and kept at 4°C for 3 days in the dark. Then plants were transferred to the conditions of a 14-h-light (80 μmol photons m⁻² s⁻¹)/10-h-dark cycle at 23°C. The AOR-deficient *Arabidopsis* mutant *aor*, which was previously identified as a T-DNA knockout line of AOR¹⁶, was obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA). HSF1A quadruple knock-out mutant (QK) was generously gifted by Dr Y.-Y. Chang, National Taiwan University, Taiwan. Seeds of rice (*Oryza sativa* L., cv. Nipponbare) were immersed in water for a day at 4°C, and the transferred to the conditions of a 14-h-light (80 μmol photons m⁻² s⁻¹)/10-h-dark cycle at 25°C. Oxidative treatment was performed by immersing the aerial parts of plants in 10 μM methylviologen (MP Biomedicals, Solon, OH, USA) under illumination (80 μmol photons m⁻² s⁻¹). UV-B treatment was performed by irradiation of UV light (VL-6MC, 312 nm tube, Vilber Lourmat, France) with 1 mW cm⁻². Heat treatment was performed by exposing at 40°C in the presence of light (80 μmol photons m⁻² s⁻¹).

Volatile treatment. Plants were placed in a transparent plastic box (340 cm³, Nippon Genetics, Tokyo, Japan). Volatiles were diluted with MeCN, which does not induce HSF1A2 mRNA. Each volatile tested (total volume of 3 μl) was absorbed into a piece of paper towel attached to the inside of the cover. The cover was immediately set on the box (Supplementary Fig. S1a), and the plants were incubated at 25°C under illumination (80 μmol photons m⁻² s⁻¹). MeCN-treated plants were used as controls.

Quantitative real-time RT-PCR (qRT-PCR). Total RNA was purified by using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and then cDNA was synthesized by using a RevaTra Ace kit (Toyobo, Osaka, Japan). Gene-specific primers were

chosen with the use of the Primer3 program (<http://frodo.wi.mit.edu/>). Primer sequences are shown in Table S2. Quantitative real-time RT-PCR (qRT-PCR) was performed with the use of Thunderbird SYBR Green qPCR Mix (Toyobo) and a LineGene Real-time PCR Detection System (FQD-33A, BioFlux, Tokyo, Japan). For analysis of relative transcript levels, *ACTIN2* mRNA was used as an internal standard in all qRT-PCR experiments; the expression levels of genes of interest were normalized to that of *ACTIN2* by subtracting the cycle threshold (CT) value of *ACTIN2* from the CT value of the gene of interest. The expression level of MeCN-treated controls was set to 1. For determination of absolute copy number of HSF1A2, we used samples including equal amount of *ACTIN2* determined by qRT-PCR and the pMD20 plasmid (Takara Bio Inc., Otsu, Japan) containing the HSF1A2 (3.6 kbp, 254 copies/fg) as a reference matrix.

Microarray RNA sample preparation and hybridizations. Total RNA was purified by using an RNeasy Plant Mini Kit (Qiagen) from at least 6 plants. The double-strand (ds) cDNA was generated with a modified procedure of the Superscript Choice System (Life Technologies, Carlsbad, CA). Briefly, the 1st strand cDNA was synthesized from 10.0 μg total RNA by 1.0 unit SuperScript II reverse transcriptase (Life Technologies) in the presence of 100 pmoles Oligo dT(20) primer. After 2nd strand synthesis, the template RNA was digested with RNase A, then the synthesized DNA was purified with phenol:chloroform:isoamyl alcohol. The purified DNA was precipitated in ethanol, and the pellet was washed, dried, reconstituted and quantified. cDNA samples were labeled using the random priming method with Cy3-labeled random nonamer as primers and Klenow DNA polymerase at 37°C for 2 h (NimbleGen One-color Labeling Kit, NimbleGen Roche, Madison, WI). The labeled DNA was precipitated in isopropanol, and the pellet was washed, dried, reconstituted and quantified. For each hybridizations, 4 μg of labeled DNA was diluted by NimbleGen sample tracking control kit buffers respectively, and be added with NimbleGen hybridization buffers according to the manufacturer’s protocols. The arrays (ATH6_60mer_expr X4) were hybridized with labeled DNA on a NimbleGen Hybridization System at 42°C for 16 h. Arrays were washed by NimbleGen wash buffer kit according to the manufacturer’s protocols and scanned using an Axon GenePix 4000B scanner at 5 μm resolution. The microarray experiments using the Agilent Arabidopsis ver4.0 (44 k) microarray (Agilent Technologies) with a one-color method were performed according to the manufacturer’s instructions. Data extraction, normalization and production of heat map by UPGMA clustering were performed by Subio Platform software (Subio, Kagoshima, Japan), and Microsoft

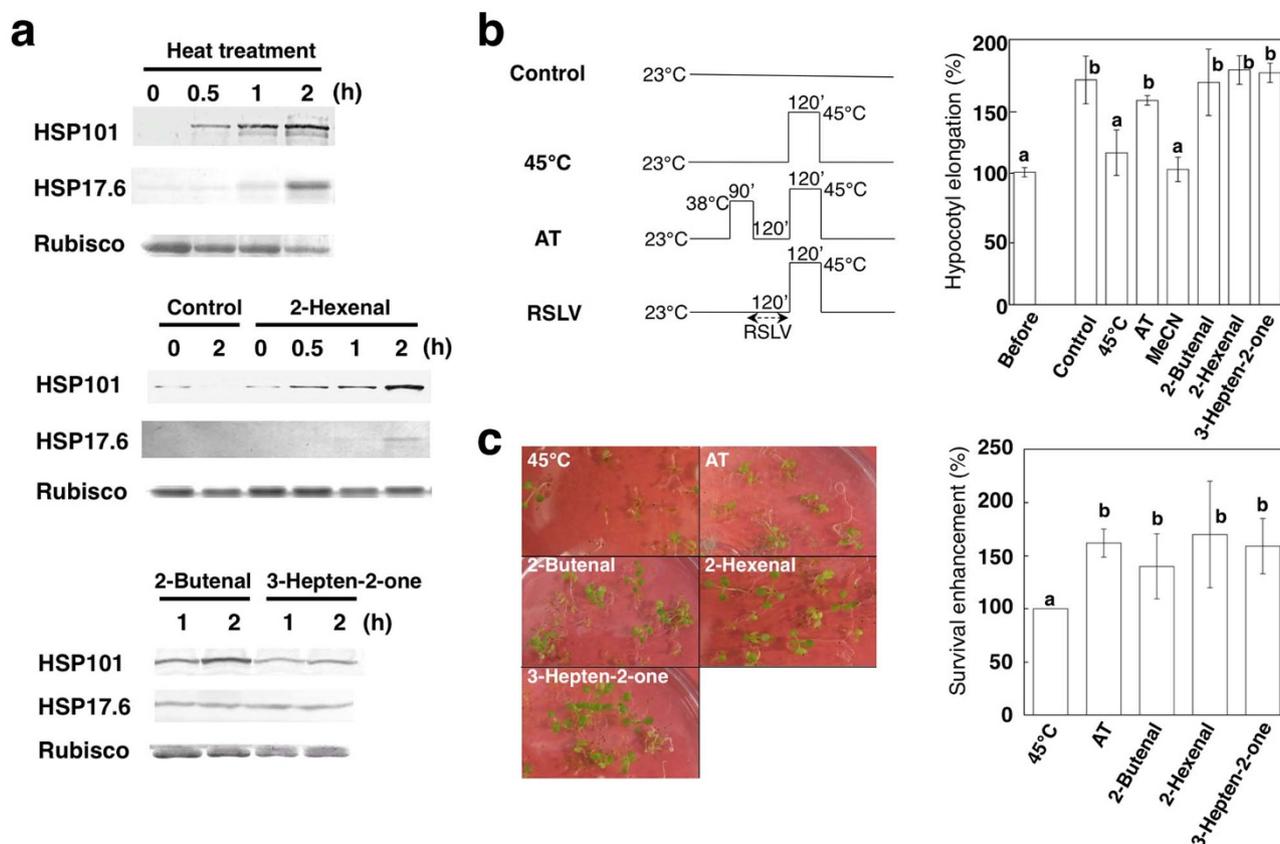


Figure 4 | Biological effects of RSLV treatment of *Arabidopsis*. (a), After the indicated RSLV treatment or heat treatment at 38°C, expression of HSP101 and HSP17.6 proteins was detected by western blot analysis. Rubisco was stained using Coomassie Brilliant Blue R-250 as a loading control. These images are cropped from original images shown in Supplementary Fig. S11. (b), The 2.5-day-old dark-grown seedlings (Before) were pretreated at 38°C for 90 min to acquire thermotolerance (AT) or 10 μM indicated RSLVs or solvent control (MeCN) for 2 h and then heat-stressed at 45°C for 2 h. Seedling were returned to 23°C in the dark and length was measured after 2.5 days. Length of seedlings before treatment was set to 100%, and elongation of each treatment was calculated. Schemes of treatment are shown above the graph. (c), Survival enhancement was calculated by survival rate determined on 3 days after treatments with same scheme as shown in panel (b). *Left*; Representative photographs of *Arabidopsis* plants on 7 days after heat stress treatments. *Right*; Survival enhancement by RSLV treatment calculated from 3 independent assays. Survival rate of 45°C sample was set to 100%. Values followed by the same letter are not significantly different according to Tukey-Kramer ($n = 4$ or 5 ; $P < 0.05$).

Excel was used to organize and interpret the data. “Ratio” values are the mean of 3 independent experiments, and genes showing a value above 2.0 or below 0.5 (99.8% confidence) were considered as up- or down-regulated, respectively. Datasets of gene expression under each stressed condition were obtained from AtGenExpress database

(The *Arabidopsis* Information Resource, The Ohio State University, OH, USA). Expression data used in this figure are data of shoot in time course (0.25, 0.5, 1, 3, 6 h of heat, UV-B, drought or wound stresses) and (0.5, 1, 3, 6, 12 h of oxidative, salt, osmotic or cold stresses).

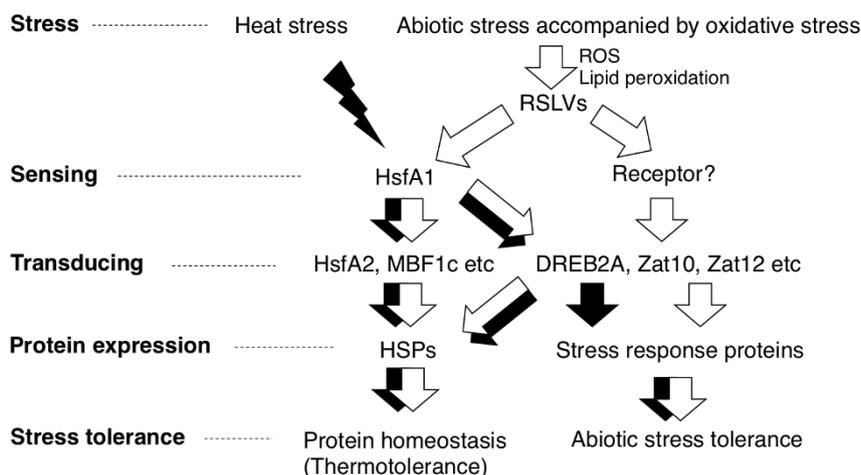


Figure 5 | Hypothesized RSLVs-related signaling pathway in abiotic stress accompanied by oxidative stress. RSLV stimulates both HSF1-dependent and –independent pathways (white arrows). RSLVs are possibly involved in the upstream of signaling pathway triggered by abiotic stresses accompanied by oxidative stress. Proteotoxic stress such as heat and ROS are accepted by HSF1s to drive HSF1-dependent pathway (black arrows).



Identification of RSLVs. Harvested plants were immersed in MeCN (2 ml), and 1 μ l of 10 mM 2-ethylhexanal was added as an internal standard. After incubation for 30 min at 60°C, 1 ml of the solution was transferred to another glass tube, and 38 μ l of HCOOH and 50 μ l of 20 mM dinitrophenyl hydrazine dissolved in MeCN were added. After incubation for 60 min at room temperature, 1 ml of saturated NaCl solution and 0.1 g of NaHCO₃ were added and well mixed. After incubation for 20 min, 0.5 ml of the MeCN layer was transferred to a plastic tube and evaporated. The resultant residue was dissolved in 200 μ l of MeCN, and the solution was filtered through a Cosmonice filter (pore size 0.45 μ m, Nacalai Tesque, Kyoto, Japan). The resultant cleared solution was used as the DNP-carbonyl preparation for HPLC analysis. HPLC analysis was performed by the method described previously^{20,31,32}. Data were analyzed by using PowerChrom software (eDAQ Pty Ltd., Denistone East, NSW, Australia).

For accurate identification of DNP-RSLVs, DNP-RSLVs were subsequently analyzed by liquid chromatography-mass spectrometry (LC/MS/MS, Acquity UPLC/TQD; Waters, Milford, MA, USA) using YMC-PAK C4 (ϕ 2.0 \times 100 mm, 3 μ m, Waters). For reverse phase chromatography of RSLV-DNPs, the elution of the samples was carried out with 10% tetrahydrofuran (solvent A2) and acetonitrile (solvent B2), and the mobile phase was changed from 35% (v/v) B2–100% (vol/vol) at 2 and 21.5 min after the injection, respectively, at a flow rate of 0.3 mL·min⁻¹. Elution was monitored by Photodiode and MS analysis with ES-negative mode. The column temperature was 40°C. MS/MS analysis conditions were as follows: Declustering potential, 40; collision energy, 40 V; and parent ion (m/z), 249 for (E)-2-butenal-DNP.

Protein analysis. Protein was extracted with 5 volumes of 50 mM HEPES-NaOH, pH 7.0. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% (HSP101) or 12% polyacrylamide gels (HSP17.6). The proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (ATTO, Tokyo, Japan) according to the manufacturer's instructions, and anti-HSP101 and anti-HSP17.6 antibodies (Agrisera, Vännäs, Sweden) were used for immunochromatological detection. Alkaline phosphatase-conjugated secondary antibody was used for visualizing signals with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates. The bands were quantified by densitometric analysis using ImageJ software after scanning the blotted membrane.

Measurement of chlorophyll fluorescence. The maximum quantum yield of PSII was estimated from chlorophyll fluorescence measurements by pulse-amplitude-modulated (PAM) fluorometer (Junior-PAM; Walz, Effeltrich, Germany). Plants were dark-adapted at room temperature for 5 min before measuring. The yield of PSII was calculated as the ratio of F_v/F_m .

Thermotolerance test. For a hypocotyl elongation test³³, seeds planted on MS plates were wrapped in foil and incubated at 4°C for 3 days, then at 23°C for 2.5 days (Col-0) or 5 days (QK). After length of cotyledons was measured, they were separately treated as follows. i) For 45°C treatment, seedlings were incubated at 45°C for 2 h. ii) For acquired thermotolerance treatment, seedlings were incubated at 38°C for 90 min followed by 2 h at 23°C and then 2 h at 45°C. ii) For volatile treatment, seedlings were treated with 10 nmol cm⁻³ of (E)-2-butenal, (E)-hexenal or 3-hepten-2-one for 2 h at 23°C and then 2 h at 45°C. After treatment, seedlings were incubated an additional 2.5 days in the dark, then length of seedlings was measured.

For survival test, seeds (at least 30 plants) planted on MS plates were wrapped in foil and incubated at 4°C for 3 days, then at 23°C for 2.5 days. After the seedlings were subjected to the same treatments as elongation test, seedlings were additionally incubated under standard conditions of a 14-h-light (80 μ mol photons m⁻² s⁻¹)/10-h-dark cycle at 23°C. The percentage of survival plants was calculated by counting the continuously developing plants per total plants after 3 days. Survival enhancement was determined by calculation using survival rate of RSLV treatment sample against that of 45°C sample.

Statistical analysis. Data were analyzed by using the programs of Statistical analysis program (StatPlus, AnalystSoft). Data were subject to ANOVA with post hoc analysis, and means were compared by Tukey-Kramer test (P < 0.05).

Gene identifier. Arabidopsis; Actin2, At3g18780; HSF2A, At2g26150; HSF1A1, At4g17750; HSF1A1b, At5g16820; HSF1A1d, At1g32330; HSF1A1e, At3g02990; HSF1B1, At4g36990; DREB2A, At5g05410; ZAT10, At1g27730; ZAT12, At5g59820; MBF1c, At3g24500.

Tomato: Actin, Sl11g005330; HSF1A1, Sl08g005170; HSF2A, Sl08g062960; HSF1B1, Sl02g090820.

Rice: Actin1, Os03g0718100; HSF2A2c, Os10g0419300; HSF2A2d, Os03g0161900; HSF2A2e, Os03g0795900; DREB2A, Os01g0165000.

- Boyer, J. S. Plant productivity and environment. *Science* **218**, 443–448 (1984).
- Mittler, R. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* **7**, 405–410 (2002).
- Foyer, C. H. & Noctor, G. Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol. Plant* **119**, 355–364 (2003).
- Miller, G., Shulaev, V. & Mittler, R. Reactive oxygen signaling and abiotic stress. *Physiol. Plant* **133**, 481–489 (2008).
- Ramel, F. *et al.* Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. *Proc. Natl. Acad. Sci. USA* **109**, 5535–5540 (2012).
- Mueller, M. J. & Berger, S. Reactive electrophilic oxylipins: Pattern recognition and signalling. *Phytochemistry* **70**, 1511–1521 (2009).
- Yamauchi, Y. *et al.* Malondialdehyde generated from peroxidized linolenic acid causes protein modification in heat-stressed plants. *Plant Physiol. Biochem.* **46**, 786–793 (2008).
- Yamauchi, Y. & Sugimoto, Y. Effect of protein modification by malondialdehyde on the interaction between oxygen-evolving complex 33 kDa protein and photosystem II core proteins. *Planta* **231**, 1077–1088 (2010).
- Vollenweider, S., Weber, H., Stolz, S., Chételat, A. & Farmer, E. E. Fatty acid ketodienes and fatty acid ketotrienes: Michael addition acceptors that accumulate in wounded and diseased *Arabidopsis* leaves. *Plant J.* **24**, 467–476 (2000).
- Alm eras, E. *et al.* Reactive electrophile species activate defense gene expression in *Arabidopsis*. *Plant J.* **34**, 205–216 (2003).
- Nishizawa, A. *et al.* Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. *Plant J.* **48**, 535–547 (2006).
- Suzuki, N. *et al.* Enhanced tolerance to environmental stress in transgenic plants expressing the transcriptional coactivator multiprotein bridging factor 1c. *Plant Physiol.* **139**, 1313–1322 (2005).
- Kie bowicz-Matuk, A. Involvement of plant C₂H₂-type zinc finger transcription factors in stress responses. *Plant Sci.* **185–186**, 78–85 (2012).
- Mizoi, J., Shinozaki, K. & Yamaguchi-Shinozaki, K. AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta* **1819**, 86–96 (2012).
- Mano, J., Tokushige, K., Mizoguchi, H., Fujii, H. & Khorobrykh, S. Accumulation of lipid peroxide-derived, toxic α , β -unsaturated aldehydes (E)-2-pentenal, acrolein and (E)-2-hexenal in leaves under photoinhibitory illumination. *Plant Biotech.* **27**, 193–197 (2010).
- Matsui, K., Sugimoto, K., Mano, J., Ozawa, R. & Takabayashi, J. Differential metabolisms of green leaf volatiles in injured and intact parts of a wounded leaf meet distinct ecophysiological requirements. *PLoS ONE* **7**, e36433 (2012).
- Matsui, K. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. *Curr. Opin. Plant Biol.* **9**, 274–280 (2006).
- Duan, H., Huang, M. Y., Palacio, K. & Schuler, M. A. Variations in CYP74B2 (hydroperoxide lyase) gene expression differentially affect hexenal signaling in the Columbia and Landsberg *erecta* ecotypes of *Arabidopsis*. *Plant Physiol.* **139**, 1529–1544 (2005).
- Yamauchi, Y., Hasegawa, A., Taninaka, A., Mizutani, M. & Sugimoto, Y. NADPH-dependent reductases involved in the detoxification of reactive carbonyls in plants. *J. Biol. Chem.* **286**, 6999–7009 (2011).
- Yamauchi, Y., Hasegawa, A., Mizutani, M. & Sugimoto, Y. Chloroplastic NADPH-dependent alkenal/one oxidoreductase contributes to the detoxification of reactive carbonyls produced under oxidative stress. *FEBS Lett.* **586**, 1208–1213 (2012).
- AH-Mackerness, S., John, C. F., Jordan, B. & Thomas, B. Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide. *FEBS Lett.* **489**, 237–242 (2001).
- Takeuchi, Y., Fukumoto, R., Kasahara, H., Sakaki, T. & Kitao, M. Peroxidation of lipids and growth inhibition induced by UV-B irradiation. *Plant Cell Rep.* **14**, 566–570 (1995).
- Liu, H. C., Liao, H. T. & Charny, Y. Y. The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in *Arabidopsis*. *Plant Cell Environ.* **34**, 738–751 (2011).
- Buttery, R. G., Ling, L. C. & Light, D. M. Tomato leaf volatile aroma components. *J. Agric. Food Chem.* **35**, 1039–1042 (1987).
- Hernandez, H. P., Hsieh, T. C. Y., Smith, C. M. & Fischer, N. H. Foliage volatiles of two rice cultivars. *Phytochemistry* **28**, 2959–2962 (1989).
- Hong, S. W. & Vierling, E. Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. *Proc. Natl. Acad. Sci. USA* **97**, 4392–4397 (2000).
- Dafny-Yelin, M., Tzfira, T., Vainstein, A. & Adam, Z. Non-redundant functions of sHSP-Clis in acquired thermotolerance and their role in early seed development in *Arabidopsis*. *Plant Mol. Biol.* **67**, 363–373 (2008).
- Loreto, F., Barta, C., Brilli, F. & Nogues, I. On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. *Plant Cell Environ.* **29**, 1820–1828 (2006).
- Copolovici, L., K nnaste, A., Pazouki, L. & Niinemets, U. Emissions of green leaf volatiles and terpenoids from *Solanum lycopersicum* are quantitatively related to the severity of cold and heat shock treatments. *J. Plant Physiol.* **169**, 664–672 (2012).
- Vickers, C. E., Gershenzon, J., Lerdau, M. T. & Loreto, F. A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nature Chem. Biol.* **5**, 283–291 (2009).
- Matsui, K., Sugimoto, K., Kakumyan, P., Khorobrykh, S. A. & Mano, J. Volatile oxylipins and related compounds formed under stress in plants, *Methods in Molecular Biology 'Lipidomics' Vol. 580* (Armstrong, D. ed.) 17–28 (Humana Press, Totowa, NJ, 2009).



32. Yin, L., Mano, J., Wang, S., Tsuji, W. & Tanaka, K. The involvement of lipid peroxide-derived aldehydes in aluminum toxicity of tobacco roots. *Plant Physiol.* **152**, 1406–1417 (2009).
33. Hong, S. W. & Vierling, E. Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. *Proc. Natl. Acad. Sci. USA* **97**, 4392–4397 (2000).

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Author contributions

Y.Y. designed the project, performed experiments and wrote the manuscript; M.K. performed experiments using *Arabidopsis* seedlings. M.M. supervised molecular biological and biochemical analyses. Y.S. supervised chemical analysis.

Additional information

Accession codes: The transcriptome expression data from RSLV-treated *Arabidopsis* seedlings have been deposited under the following accession codes: GSM1569068-1569075.

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