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Original Paper

Multiple hormone treatment revealed novel cooperative relationships between abscisic acid and biotic stress hormones in cultured cells

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Abstract Phytohormones have vigorous crosstalk relationships. For example, abscisic acid (ABA), a hormone involved in abiotic stress responses, has antagonistic interactions with plant hormones that play pivotal roles in defense responses, including salicylic acid (SA) and methyl-jasmonic acid (MeJA). Evidence indicates that the relationships among these plant hormones extend beyond simple antagonism. To explore the interplay between hormones in detail, we analyzed the effects of double hormone treatment on gene expression. By contrast to the antagonistic effects reported previously, our data indicates that ABA interacts with SA and MeJA cooperatively as well. Particularly many genes responded only to double hormone treatment, and, interestingly, the loci that responded to ABA+SA also responded to ABA+MeJA. The expression of early-response genes following double hormone treatment did not fit the linear superposition of individual hormone treatments, in contrast to mammalian and prokaryotic cell responses to multiple chemical stimuli. Thus, synergies in these plant hormone signalings are not simply the sum of individual responses. ABA and SA collaboratively down-regulated the expression of genes involved in cell cycle progression at G2/M phase. Presumably, plants interpret combined hormone signals differently from individual signals in order to respond appropriately to their environmental conditions.

Key words: Abscisic acid, Arabidopsis cultured cell, jasmonic acid, salicylic acid, transcriptome.

Plants utilize phytohormone signaling systems to maintain their cellular and whole-body functions. Endogenously synthesized chemicals, including auxin, cytokinin, gibberellins, abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and brassinosteroids, are broadly accepted as phytohormones (Santner et al. 2009). Strigolactones were recently identified as phytohormones (Gomez-Roldan et al. 2008; Umehara et al. 2008), suggesting that additional plant growth regulators await discovery. Phytohormones participate in diverse fundamental physiological processes, including developmental regulation and stress responses. Accordingly, there have been extensive explorations of the molecular mechanisms underlying phytohormone actions for more than a century. These efforts led to the determination of signaling pathways from perception to gene regulation for most plant growth regulators (Santner and Estelle 2009). Soluble ABA receptors were identified recently (Ma et al. 2009; Park et al. 2009), allowing us to establish the main signaling pathway of this hormone (Fujii et al. 2009; Umezawa et al. 2009).

As our understanding of the physiological functions of phytohormones has deepened, complicated crosstalk among phytohormones has become evident, particularly among ABA, SA, JA, and ET, which are involved in abiotic and biotic stress responses. Such crosstalk has received significant attention recently because it likely plays important roles in the coordination of stress responses under natural conditions. Research has uncovered complicated (though still controversial) relationships among these hormones (Mauch-Mani and Mauch 2005). For example, ABA plays a key role in defense responses through complicated collaborations with other plant hormones, including SA, JA, and ET (Asselbergh et al. 2008; Fan et al. 2009; Ton et al. 2009;

Abbreviations: ABA, abscisic acid; ET, ethylene; MeJA, methyl jasmonate; PCR, polymerase chain reaction; SA, salicylic acid.
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de Torres-Zabala et al. 2007). Despite the importance of phytohormone crosstalk, the underlying molecular mechanisms have yet to be elucidated. There is little information on the cellular responses to multiple hormone signals (i.e., the product of this crosstalk). To date, there has been no systematic analysis of the way in which plant cells respond to multiple hormone treatments. Moreover, many studies of hormone crosstalk have used whole plants or tissues, which are composed of many different types of cells, as their experimental material. It is possible that different mature cell types have distinct responses to hormones. Supporting evidence for this hypothesis is provided by the differential responses among different types of root cells to abiotic stress (Dinneny et al. 2008). Simplified experimental systems are likely the best option for improving our understanding of the mechanisms underlying these complicated interactions.

Cellular responses to multiple stimuli are of considerable current interest. Treatments for multi-factor diseases require information on appropriate combinations and doses of drugs. Progress on this issue ultimately requires an exploration of the molecular bases of cellular responses. Protein level changes in response to multiple conflicting chemical stimuli have been investigated systematically in animal cells and *Escherichia coli* (Bollenbach and Kishony 2011; Geva-Zatorsky et al. 2010). These investigations showed that protein level responses to combinations of chemicals are basically linear superpositions of the responses to individual chemicals. Equivalent data are not available for plant systems; thus, it is unclear whether the conclusions for mammals and prokaryotes can be extrapolated to vascular plants.

We previously reported changes in metabolite levels in cultured *Arabidopsis* cells treated with ABA and SA. The overall metabolite profiles demonstrated that ABA and SA do not act antagonistically; instead, they operate additively (Okamoto et al. 2009). To improve our understanding of the fundamental bases for the interactions between abiotic and biotic stress hormones, we comprehensively analyzed the transcript levels in cultured *Arabidopsis* cells treated with ABA, SA, and methyl-jasmonate (MeJA) in various combinations. We found that ABA and SA had both antagonistic and additive effects on gene expression. A detailed analysis of the genes affected by ABA+SA treatment demonstrated that these hormones cooperatively regulate several genes. Moreover, there were correlations between the responses to ABA+SA and ABA+MeJA. The expression values of most genes that were regulated in the early stages of combined hormone treatment did not fit clearly linear superpositions of their individual stimulus values; hence, the general model for mammalian cells and *E. coli* cannot be extrapolated to plant hormone response. We also found that SA strengthened the ABA-induced inhibition of genes involved in cell cycle progression at G2/M phase; combined treatment with these hormones induced a cell cycle arrest. We discuss the physiological relevance of these responses and the usefulness of cultured cells in hormone crosstalk studies.

Materials and methods

Plant materials and sample preparation

T87 *Arabidopsis thaliana* cells obtained from the RIKEN BioResource Center (Tsukuba, Japan) were incubated and subcultured every 7 days in 20 ml of liquid-modified JPL medium (Axelos et al. 1992) in a 100-ml baffled Erlenmeyer flask mixed at 100 rpm on a rotary shaker at 24°C under a 16 : 8 h light/dark cycle. Prior to inoculation, 55 mg of cells (wet weight) were filtered through a 2-mm nylon mesh. For plant hormone treatment, 20 ml of 6-day-old cultured cells were incubated in the presence of hormone (25 μM ABA, 300 μM SA, and 100 μM MeJA). Next, the cells were washed with distilled water, frozen immediately in liquid N_2, and stored at –80°C. To estimate the nuclear DNA content, the cells were incubated for 48 h with the above hormones. The DNA contents of the cells were measured using previously described methods (Yoshizumi et al. 2006).

DNA microarray analysis

Total RNA was isolated with Trizol reagent (Invitrogen Japan KK, Tokyo, Japan) and purified using an RNeasy purification kit (Qiagen KK, Tokyo, Japan). cDNA synthesis, cRNA synthesis, and hybridization to the Affymetrix ATH1 Genome Array were performed according to the manufacturer's recommendations (Affymetrix KK, Tokyo, Japan). The experiments were duplicated using different cell culture lots. The microarray data were processed with affylmGUI running in the R software environment (Wettenhall et al. 2006). The robust multiarray analysis (RMA) algorithm was used for background correction, normalization, and to summarize expression (Irizarry et al. 2003). Differential expression analysis was performed with Bayes t-statistics using linear models for microarray data (Limma) within the affylmGUI software package. *P*-values were corrected for multiple testing using the Benjamini-Hochberg method (false discovery rate) (Reiner et al. 2003). Probe sets with a $\geq$2-fold response and a *P*-value $<$0.05 for at least one hormone treatment were collected and used further analysis (total: 6998 probe sets; after removing probe sets for organelle genes and no corresponding genes: 6903 probe sets; Table S1). Our microarray data were deposited in the NCBI GEO database under accession number GSE28600. For clustering analysis and heat map plotting, we used Genevestigator (https://www.genevestigator.com/gv/) (Zimmermann et al. 2004) and MeV (v4.3) (Saeed et al. 2003) software. Hierarchical analysis and linear regression were performed with the R software package. *Cis*-regulatory element searching was performed with AtCOESIS software (http://bioinformatics.psb.ugent.be/

Table 1. Expression of hormone responsive genes

Locus	3 h		24 h		Description
	Exp[a]	p value	Exp[a]	p value	
ABA-responsive genes					
AT3G02480	8.55	6.00E-06	9.00	2.67E-06	ABA-responsive protein-related
AT5G52310	7.61	7.09E-07	4.65	7.40E-05	COR78
AT5G06760	7.02	5.24E-09	6.58	1.40E-08	Group1 LEA protein
AT5G66400	5.95	2.04E-07	8.85	3.07E-09	RAB18
AT5G59220	5.58	1.93E-06	5.25	2.94E-06	HAI1 (PP2C)
AT5G57050	4.93	3.91E-06	3.29	0.0002	ABI2 (PP2C)
SA-responsive genes					
AT5G22570	8.69	3.19E-06	7.95	2.93E-06	WRKY38
AT3G56400	4.11	5.63E-06	4.53	6.54E-07	WRKY70
AT1G02450	3.53	3.19E-06	5.31	4.05E-09	NIMIN-1/NIMIN1
AT5G45110	3.07	0.0014	2.53	0.0057	NPR3
AT2G43820	3.63	0.0014	4.29	7.99E-05	UDP-Glucosyltransferase 74F2
AT5G67160	2.99	4.02E-05	2.82	3.56E-05	EPS1 (tranferase)
JA-responsive genes					
AT3G55970	8.49	1.12E-08	7.60	1.01E-07	2OG-Fe(II) oxygenase family
AT5G13220	5.75	6.21E-09	5.00	6.30E-08	JAS1/JAZ10/TIFY9
AT5G24770	5.44	4.81E-05	7.73	5.64E-07	VSP2
AT3G16450	5.12	6.69E-05	7.87	3.65E-07	jacalin lectin family protein
AT1G44350	4.58	8.04E-06	4.85	3.73E-06	ILL6
AT1G17380	3.20	3.60E-07	2.59	4.82E-06	JAZ5/TIFY11A

a, $\log_2$ expression ratio (treatment/control), the mean of two independent biological experiments.

ATCOECIS/) (Vandepoele et al. 2009).

Real-time RT-PCR

Total RNA was isolated from cultured cells using TRIzol extraction reagent (Invitrogen Japan KK). cDNA was synthesized from 1 μg of total RNA using a ReverTra Ace Kit (Toyobo, Osaka, Japan). Real-time PCR was performed on a LightCycler (Roche Diagnostics, Mannheim, Germany) in a total volume of 20 μl containing 10 μl of SYBR Green Real-Time PCR Master Mix -Plus- (Takara Bio Inc., Otsu, Japan), 8 pmol of each primer, and 1/40 of the cDNA mixture. The amplification program consisted of 50 cycles of 95°C for 10 s and 60°C for 1 min. We used the comparative Ct method with *ACTIN2* as a control. We used the primers At1g44110F, 5′-GGCTTATTGAGGTGTCTGAAGAG-3′; At1g44110R, 5′-ACAAGCCACACCAAGCAACT-3′; At3g11520F, 5′-GTTGTGAATGAGAGCAAACCTCAG-3′; At3g11520R, 5′-AGGGGAGAGATCAAACTTGACA-3′; At4g31840F, 5′-CGGCGAATTTATCGTATTCAAG-3′; At4g31840R, 5′-TGGCTTTAGGGCTAGTGGTG-3′; At1g20930F, 5′-TCATGGAGTGTTGCACAGGGATC-3′; At1g20930R, 5′-TTGGGAGAGTGAAGGCTCTG-3′; ACTIN2F, 5′-GAGGATGGCATGAGGAAGAGAGAAAC-3′; and ACTIN2R, 5′-GCCAGTGGTCGTACAACCGGTATT-3′.

Results

Overall transcript profile following single hormone treatment

We first evaluated our experimental procedures using cultured cells and single hormone treatments (see Materials and methods). ABA caused dramatic changes in gene expression, consistent with data produced using intact *Arabidopsis* seedlings (Goda et al. 2008). The loci up-regulated by ABA included stress-inducible genes such as *RAB18*, *COR78*, *LEA*, and genes for PP2Cs (Table 1). SA-up-regulated genes included several known SA-inducible loci, including those encoding the transcription factors WRKY38, WRKY70, and NPR1-interacting protein (NIMIN-1) (Li et al. 2004; Wang et al. 2006; Weigel et al. 2001). *UGT74F2* encoding the SA metabolic enzyme was also up-regulated (Song 2006), consistent with previous data (Okamoto et al. 2009). Treatment with MeJA also affected the mRNA level of many genes in the cultured cells, including the known MeJA-inducible genes *VSP1* and *ILL6*, and genes encoding several JAZ-type transcription factors (Table 1) (Ma et al. 2006).

The inducible expression of several ABA- or SA-responsive genes was reported to be repressed by the antagonistic interaction of the other hormone (Yasuda et al. 2008). In this study, indeed, several ABA- or SA-inducible genes were repressed by treatment with the other hormone (Table S2). For example, *At1g03850*, which encodes a glutaredoxine, was strongly up-regulated by 3 h of treatment with SA (3.48 in $\log_2$ ratio, $P=5.63E^{-6}$), but down-regulated by 3 h of treatment with ABA (–1.41 in $\log_2$ ratio, $P=0.0018$). However, the proportion of such genes in the set was quite small. ABA and MeJA also have antagonistic relations (Anderson et al. 2004; Ton et al. 2009)(Table S2). *VSP1*, as indicated

above, was strongly up-regulated by 3 h of treatment with MeJA (5.44 in $\log_2$ ratio, $P=4.81E^{-5}$) but down-regulated by 3 h of treatment with ABA (–1.68 in $\log_2$ ratio, $P=0.0346$). These data are consistent with previous results showing that ABA and JA function, at least partly, antagonistically.

Effects of ABA+SA

As indicated above, our transcriptome data demonstrated that our experimental procedures were sufficiently reliable. Thus, we examined the early and late responses of cultured plant cells stimulated simultaneously with ABA and SA. Figure 1A depicts the result of a clustering analysis using genes whose transcript levels were up- or down-regulated ≥2 fold ($P<0.05$) by ABA and/or SA (5870 genes). The overall expression profile of most of the genes that responded to ABA+SA was broadly similar to that of the genes that responded to ABA only; the expression of other genes in response to ABA+SA more closely resembled that following SA treatment. Remarkably, ABA+SA had unique marked effects on gene expression.

We analyzed the expression profiles following ABA+SA treatment in greater detail. Those transcripts whose levels changed ≥2 fold ($P<0.05$) following treatment with ABA, SA, or ABA+SA were selected and further classified by their levels in each treatment (Figure S1A). Using this procedure, we classified 1873, 1265, 2559, and 1644 genes into the following four categories: those up-regulated by 3 h of treatment, those up-regulated by 24 h of treatment, those down-regulated by 3 h of treatment, and those down-regulated by 24 h of treatment, respectively. Surprisingly, among the SA-regulated genes, a considerable number were up- or down-regulated by ABA and ABA+SA [Figure S1A: sections (a), (c), and (e) in each Venn diagram]. These data indicate that ABA and SA similarly regulate the expression of a group of genes.

Interestingly, there were many ABA+SA-specific genes (up- or down-regulated ≥2 fold by ABA+SA but <2 fold by ABA or SA treatment). After 3 h of treatment, 641 and 1007 genes were up- and down-regulated, respectively, by ABA+SA [Figure S1A–D, section (f)]. Such gene responses have not been reported previously. It appears that ABA and SA act cooperatively to regulate the expression of those genes. The number of genes regulated only by 24 h of treatment with ABA+SA was less than those by 3 h, suggesting that the effects of double hormone treatment were rather transient. To explore this hypothesis, we selected genes affected only by 3 h of treatment with ABA+SA [Figure S1A, C: section (f)] and examined their expression levels after 24 h (Figure S1I, J). Among 641 genes up-regulated by 3 h of treatment, only 130 were up-regulated by 24 h of treatment. Among these 130 genes, 108 and 14 were

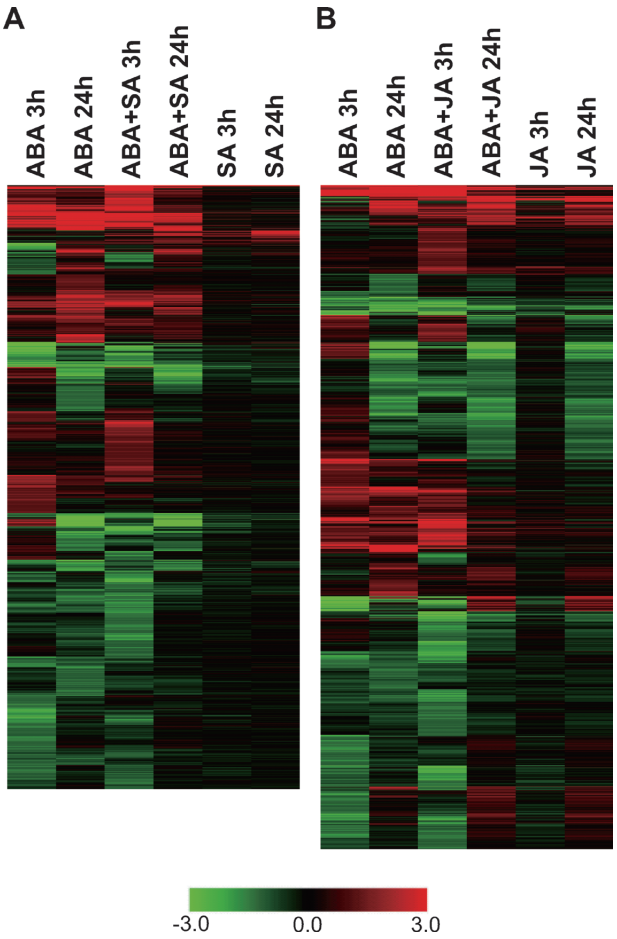

Figure 1. Clustering analysis of the genes affected by ABA, SA, and MeJA. Clustering analysis of genes up- or down-regulated ≥4 fold by treatment with ABA, SA, MeJA, ABA+SA, and ABA+MeJA for 3 or 24 h using MeV (v4.30) software. The values in the scale bar are $\log_2$ transformations.

up-regulated following treatment with ABA and SA, respectively. Fifteen other genes were classified as loci up-regulated only by ABA+SA treatment. Similarly, among 1007 genes down-regulated by 3 h of treatment, only 259 were down-regulated after 24 h of treatment. Among these 259 genes, 251 were affected by ABA or SA, and 8 were down-regulated only by ABA+SA. Thus the ABA+SA-specific effect is rather transient.

Effect of ABA+MeJA

ABA and JA interactively affect a range of plant physiological processes. To examine the effects of these hormones on gene expression, we conducted transcriptomic analyses of cells treated simultaneously with ABA and MeJA; the experimental procedures were the same as those used for ABA+SA. Genes that were up- or down-regulated ≥2 fold ($P<0.05$) by treatment with ABA, MeJA, or ABA+MeJA were collected (6669 loci) and their transcript levels were compared (Figure 1B). The overall expression profile following 3 h of treatment with ABA+MeJA was similar to that following

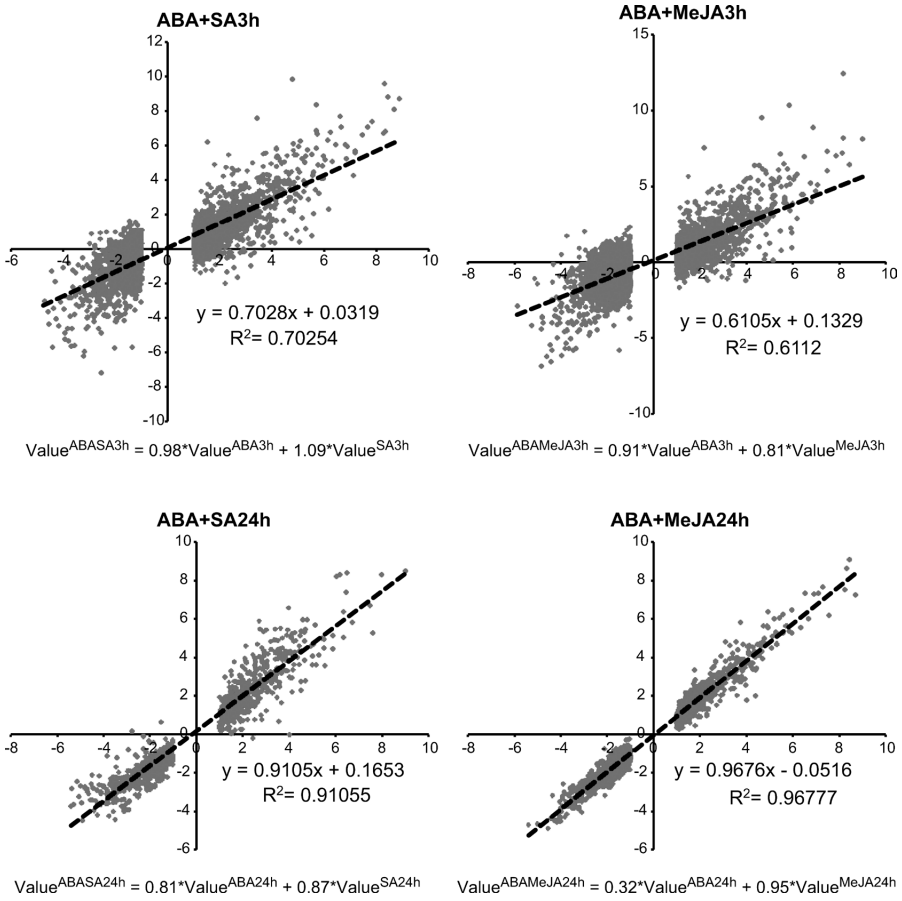

Figure 2. Correlations between the expression values for the double hormone treatments and superimposed expression values deduced from single hormone treatments. The expression values for double hormone treatment plotted against expression values calculated from the expression value fit linear superpositions of single hormone treatments. In each panel, values on the X-axis are calculated expression values for single hormone treatments; the Y-axis values are the observed expression values for double hormone treatment. The weights deduced from linear regression models are shown in the equations beneath each scatter plot. Genes whose expression was up- or down-regulated ≥2 fold ($P<0.05$) were selected (ABA+SA 3 h, 3582 genes; ABA+SA 24 h, 894 genes; ABA+MeJA 3 h, 2813 genes; ABA+MeJA 24 h, 1642 genes) and used in our calculations.

treatment with ABA. The profile following 24 h of treatment with ABA+MeJA was similar to that following MeJA treatment. This is different from the effect of treatment with ABA+SA, suggesting different crosstalk manners among different pairs of hormones.

As in our analysis of ABA+SA treatment, genes up- or down-regulated ≥2 fold by ABA and/or MeJA were selected, classified by their expression levels, and represented using Venn diagrams (Figure S1E–H). The expression of many genes was altered by both ABA and MeJA [sections (a) and (c)]. Remarkably, after 24 h, a significant number of genes were up- or down-regulated by ABA, MeJA, and ABA+MeJA treatments; 281 and 559 were up- and down-regulated, respectively [Figure S1F, H, section (a)], suggesting that ABA and MeJA coordinately regulate greater number of genes than ABA and SA do. The number of genes affected by 3 h of treatment with ABA+MeJA exceeded the number affected by 24 h of treatment, again suggesting that the double hormone effect was transient, as for ABA+SA treatment. We further examined in 24-h experiments the expression of those genes up- or down-regulated by 3 h of ABA+MeJA treatment. Among 832 and 1542 loci up- and down-regulated, respectively, by double hormone treatment [Figure S1E, G, section (f)], 220 and 620 were detected in our 24-h experiments, respectively (Figure S1K, L). Most genes up-regulated by 3 h of treatment with ABA+MeJA were also affected by 24 h of ABA treatment, similar to treatment with ABA+SA. By contrast, among the genes down-regulated by 3 h of treatment with ABA+MeJA, a larger proportion were strongly affected by 24 h of treatment with MeJA (294 among 620 genes) (Figure S1K, L).

Relationships between single and combined hormone treatments

To examine the effects of double hormone treatment more closely, genes whose expression differed ≥4 fold ($P<0.05$) between the single and double hormone treatments were selected. Few genes were selected for comparisons between SA and ABA+SA and between MeJA and ABA+MeJA. For the comparisons between

ABA and ABA+SA or ABA and ABA+MeJA, we selected 88 and 137 genes, respectively. Among these, 43 and 80 genes, respectively, were oppositely regulated by single or double hormone treatment, suggesting that a considerable proportion of the genes were significantly affected by the presence of two hormones that usually act antagonistically. Surprisingly, many of the affected genes responded to both ABA+SA and ABA+MeJA. In total, 55 genes were significantly affected after 3 h by SA and MeJA in the presence of ABA (Table S3). The expression levels of these genes did not seem to be affected by treatment with SA or MeJA alone. Thus, paired hormone treatments have distinct signaling effects on some genes.

Gene and protein expression changes in response to multiple conflicting chemical stimuli have been investigated systematically in animal cells and *E. coli* (Bollenbach and Kishony 2011; Geva-Zatorsky et al. 2010). In these cases, the gene expression changes in response to combinations of chemicals were linear superpositions of the responses to the individual chemicals: $P_{i+j} = w_i P_i + w_j P_j$, where P_{i+j} is the gene expression level given the combination of i and j stimuli, P_i and P_j are gene expression levels in the presence of either stimulus, and w_i and w_j are weights. Usually the weights are <1 and $w_i + w_j \approx 1$; these values differ among genes or proteins, depending on the strengths of the stimuli (Bollenbach and Kishony 2011; Geva-Zatorsky et al. 2010). Accordingly, we determined whether the effects of the combined phytohormone treatments could be described simply as linear superpositions of separate hormone effects. We deduced weights from the expression values for those genes whose levels more than doubled following double hormone treatment. A linear regression model was used to obtain the weights. Using the deduced weights, we calculated the correlations between the expression values for the double hormone treatments and analyzed the values calculated using the deduced weights. As shown in Figure 2, there were clear correlations between the 24-h ABA+SA and ABA+MeJA treatments. The fits were not as good for the 3-h treatments. Hence, in late or steady-state treatment responses, the expression levels of most genes were determined by the presence of a combination of plant hormones and the combined effects were no more than the sums of each hormone response. This was not the case for early responses to the treatments; the gene expression levels were likely determined differently from the individual hormone effects.

Genes regulated only by double hormone treatment

Next, we asked whether there were genes whose expression was changed only by combined hormone treatments. We searched for genes that were up- or down-regulated $\geq$2 fold after 3 and 24 h of combined hormone treatments ($P<0.05$) and affected <0.4 (in $\log_2$ ratio) by single hormone treatments. Remarkably, 110 up-regulated and 98 down-regulated genes were affected only by ABA+SA (top 25 genes are listed in Table 2), and 93 up-regulated and 118 down-regulated genes were affected only by ABA+MeJA (top 25 genes are listed in Table 3). Most of these genes were up-regulated after 3 h (only 3 were up-regulated and 1 down-regulated after 24 h of treatment), indicating that the double hormone-specific effect on gene regulation is transient.

The expression profiles of the top 50 loci (25 up-regulated and 25 down-regulated) for the double hormone-specific genes were compared with microarray data from a public database using Genevestigator (Zimmermann et al. 2004). These genes were generally up- or down-regulated by ABA treatment, consistent with our observation that most double hormone-responsive genes [Figure S1A–H, section (f)] were affected most strongly by ABA after 24 h (Figure S1I–L). Therefore, many of the ABA+SA- or ABA+MeJA-specific genes may respond to ABA following more protracted treatment. These genes are affected by several biotic and abiotic stresses, including challenges from pathogens, drought, and salinity. Interestingly, many of the loci were inversely regulated by light and sugar treatment, implying that they are involved in responses to environmental cues. The expression of these genes differed among the results in the public database, suggesting that they are sensitive to the developmental stage or experimental conditions.

Relationship between ABA+SA and ABA+JA

A comparison of Tables 2 and 3 shows that many genes were up- or down-regulated by both ABA+SA and ABA+MeJA treatments. Those genes affected by these two double hormone treatments are listed in Table 4. We determined whether these tendencies could be found in a broader group of loci. In total, 3472 up- and 4274 down-regulated genes ($\geq$2 fold by any treatment) were selected, and relationships among the treatments were deduced by a hierarchical analysis of the gene expression values of these genes (Figure 3). The gene expression profiles following 3 h of treatment with ABA+SA and ABA+MeJA were clustered closely together, implying that the two treatments evoke similar physiological responses that are different from the responses to the individual hormones. We searched for common *cis*-regulatory elements among the genes listed in Table 4 and found that ABA-responsive elements and DRE-like elements were present in the predicted promoter regions of these up-regulated genes; light-responsive elements were present in the predicted promoter regions of the down-regulated genes, consistent with the expression patterns of these genes in the public database.

Table 2. Genes up- or down-regulated by only ABA+SA treatments (top25).

Locus	Expression ratio[a]						Description
	ABA+SA		ABA		SA		
	3 h	24 h	3 h	24 h	3 h	24 h	
Up-regulated genes							
AT1G20180	**4.73**	0.16	0.22	0.08	0.38	0.14	similar to unknown protein
AT2G24762	**3.29**	−0.06	0.01	−0.02	0.32	0.17	ATGDU4 (GLUTAMINE DUMPER 4)
AT5G45630	**3.15**	0.00	0.21	0.13	0.09	−0.14	similar to unknown protein
AT4G18980	**3.11**	0.29	0.25	0.34	0.08	−0.04	similar to unknown protein
AT3G03530	**2.90**	0.12	0.10	0.23	−0.08	0.15	NPC4
AT1G79900	**2.73**	0.46	0.30	0.40	0.24	−0.03	ATMBAC2/BAC2
AT1G54130	**2.42**	0.33	0.06	−0.05	−0.26	0.01	RSH3 (RELA/SPOT HOMOLOG 3)
AT3G10320	**2.26**	0.26	0.13	0.09	0.08	0.06	similar to unknown protein
AT2G25090	**2.21**	0.01	0.22	0.22	0.39	0.02	CIPK16 (SnRK3.18)
AT1G63380	**2.16**	0.48	−0.18	−0.17	−0.05	−0.20	short-chain dehydrogenase family
AT1G02340	**2.16**	−0.18	0.20	0.39	0.34	−0.14	HFR1
AT5G54870	**2.07**	0.31	0.30	0.14	0.24	0.03	similar to unknown protein
AT1G52080	**2.07**	0.69	0.03	0.27	0.38	−0.13	AR791; actin binding
AT5G24570	**2.04**	−0.03	−0.17	0.30	−0.23	−0.07	unknown protein
AT4G32250	**2.00**	0.14	−0.26	−0.18	−0.08	−0.09	protein kinase family protein
AT3G08870	**1.98**	0.33	0.01	−0.26	−0.13	−0.02	lectin protein kinase, putative
AT5G64230	**1.97**	0.09	−0.05	0.15	0.11	−0.27	similar to unknown protein
AT5G62540	**1.86**	0.29	−0.10	−0.15	0.38	0.19	UBC3
AT5G50170	**1.85**	0.27	0.19	0.32	−0.30	−0.18	C2 domain-containing protein
AT3G22830	**1.81**	0.08	0.23	0.38	0.35	0.04	AT-HSFA6B
AT1G73220	**1.78**	−0.37	0.18	0.11	0.24	0.03	ATOCT1
AT1G54710	**1.77**	−0.01	0.13	−0.20	0.26	−0.13	AtATG18h
AT5G64210	**1.75**	−0.07	0.18	0.12	0.06	0.05	AOX2 (alternative oxidase 2)
AT3G11660	**1.72**	0.26	−0.02	0.39	−0.39	−0.32	NHL1 (NDR1/HIN1-like 1)
AT2G31260	**1.65**	0.01	−0.13	−0.17	0.01	−0.05	APG9 (AUTOPHAGY 9)
Down-regulated genes							
AT5G10820	**−1.55**	0.39	0.38	0.00	0.05	−0.05	transporter family protein
AT1G06390	**−1.55**	−0.37	0.36	−0.22	0.11	0.00	ATGSK1
AT2G02740	**−1.56**	0.13	−0.15	−0.19	0.34	0.05	ATWHY3/PTAC11
AT1G73940	**−1.58**	−0.29	−0.26	−0.36	−0.31	−0.06	similar to unknown protein
AT5G66680	**−1.60**	−0.26	−0.28	−0.33	0.31	0.21	DGL1 (defective glycosylation 1)
AT3G02630	**−1.62**	0.55	−0.24	0.36	−0.25	−0.28	acyl-(acyl-carrier-protein) desaturase
AT3G22330	**−1.62**	0.18	0.35	−0.33	−0.15	0.26	Putative Mitochondria RNA helicase
AT3G11630	**−1.62**	−0.03	−0.29	−0.28	0.22	−0.14	2-cys peroxiredoxin (BAS1)
AT4G22300	**−1.66**	0.21	−0.24	0.16	−0.16	0.31	SOBER1, carboxylesterase
AT3G53560	**−1.66**	−0.10	−0.33	−0.21	−0.16	−0.25	chloroplast lumen common family
AT2G14880	**−1.67**	−0.61	−0.01	−0.23	−0.40	−0.20	BAF60b domain-containing protein
AT2G35040	**−1.67**	0.02	0.14	0.15	−0.09	0.09	AICARFT/IMPCHase family
AT5G27990	**−1.70**	−0.05	−0.29	−0.17	0.12	0.25	similar to unknown protein
AT3G58660	**−1.81**	−0.16	0.15	−0.19	−0.26	0.12	60S ribosomal protein-related
AT3G51670	**−1.81**	−0.48	0.11	−0.26	−0.22	−0.13	SEC14 cytosolic factor family
AT3G48730	**−1.82**	−0.12	−0.37	−0.09	−0.23	−0.14	GSA2
AT3G50410	**−1.88**	0.10	−0.34	−0.15	−0.20	0.36	OBP1 (OBF BINDING PROTEIN 1)
AT4G36660	**−1.90**	−0.13	0.17	0.37	0.04	−0.14	similar to unknown protein
AT5G48580	**−1.92**	−0.28	−0.30	−0.10	−0.28	−0.02	FKBP15-2
AT1G19520	**−2.00**	−0.07	0.13	0.37	−0.39	0.08	NFD5
AT5G37310	**−2.01**	0.12	0.10	−0.19	−0.13	−0.15	transporter
AT1G23080	**−2.13**	0.65	−0.30	0.32	−0.24	−0.19	PIN7 (PIN-FORMED 7)
AT3G54080	**−2.19**	−0.45	−0.07	−0.28	−0.40	−0.30	sugar binding
AT1G36060	**−2.43**	−0.03	0.07	0.34	−0.15	−0.24	AP2 transcription factor
AT2G27810	**−2.72**	−0.23	−0.18	−0.26	−0.18	−0.10	xanthine/uracil permease family

a, $\log_2$ expression ratio (treatment/control), the mean of two independent biological experiments.

Table 3. Genes up- or down-regulated by only ABA+MeJA treatments (top25).

Locus	Expression ratio[a]						Description
	ABA+MeJA		ABA		MeJA		
	3 h	24 h	3 h	24 h	3 h	24 h	
Up-regulated genes							
AT1G20180	**4.77**	0.33	0.22	0.08	0.03	0.29	similar to unknown protein
AT4G18980	**3.35**	0.50	0.25	0.34	0.11	0.10	similar to unknown protein
AT3G61930	**3.26**	0.00	0.08	0.19	0.01	0.26	unknown protein
AT2G24762	**3.21**	0.35	0.01	−0.02	0.01	−0.09	ATGDU4 (GLUTAMINE DUMPER 4)
AT1G79900	**3.03**	0.60	0.30	0.40	0.12	0.20	ATMBAC2/BAC2
AT3G10320	**2.80**	0.20	0.13	0.09	0.33	−0.15	similar to unknown protein
AT4G32250	**2.41**	0.36	−0.26	−0.18	−0.17	0.16	protein kinase family protein
AT1G68610	**2.38**	0.40	0.13	0.26	0.04	0.00	similar to unknown protein
AT4G26700	**2.37**	0.05	0.26	0.32	0.24	0.02	ATFIM1
AT2G46030	**2.21**	0.13	0.21	−0.23	0.02	−0.27	UBC6
AT5G45630	**2.17**	0.16	0.21	0.13	−0.14	−0.03	similar to unknown protein
AT3G11660	**2.10**	0.64	−0.02	0.39	0.31	0.25	NHL1 (NDR1/HIN1-like 1)
AT1G73220	**2.08**	0.20	0.18	0.11	0.05	0.19	ATOCT1
AT5G50170	**1.99**	−0.11	0.19	0.32	−0.07	0.12	C2 domain-containing protein
AT5G24870	**1.96**	0.36	−0.22	−0.20	0.06	−0.35	C3HC4-type RING finger
AT5G07730	**1.94**	0.28	0.02	−0.02	0.25	0.22	similar to unknown protein
AT1G70610	**1.83**	−0.07	0.14	−0.15	0.19	−0.19	ATTAP1
AT1G07150	**1.83**	−0.08	0.31	−0.21	0.16	−0.16	MAPKKK13
AT2G31260	**1.83**	0.08	−0.13	−0.17	−0.12	0.05	APG9 (AUTOPHAGY 9)
AT3G47640	**1.79**	−0.29	0.29	0.17	0.14	−0.32	bHLH family protein
AT1G63380	**1.78**	0.15	−0.18	−0.17	−0.05	−0.30	short-chain dehydrogenase
AT2G39890	**1.71**	0.33	0.16	0.19	0.17	−0.01	ProT1
AT5G65205	**1.67**	0.09	−0.09	−0.09	−0.16	0.16	short-chain dehydrogenase
AT1G74080	**1.62**	0.00	0.20	0.10	0.17	0.22	MYB122
AT3G11840	**1.55**	0.49	−0.29	−0.33	−0.03	0.39	U-box domain-containing protein
Down-regulated							
AT2G24170	**−1.65**	−0.75	−0.08	0.09	−0.15	−0.33	endomembrane protein 70
AT5G23290	**−1.65**	−0.42	−0.07	−0.34	0.08	−0.18	c-myc binding protein, putative
AT5G66680	**−1.65**	−0.38	−0.28	−0.33	0.35	−0.03	DGL1 (defective glycosylation 1)
AT5G60430	**−1.65**	−0.45	0.37	0.38	−0.24	−0.24	antiporter/drug transporter
AT1G52420	**−1.66**	−0.15	0.33	0.34	0.24	0.16	glycosyl transferase family
AT1G35680	**−1.67**	−0.70	−0.38	−0.35	−0.03	−0.37	50S ribosomal protein L21
AT1G73940	**−1.67**	−0.87	−0.26	−0.36	0.19	−0.27	similar to unknown protein
AT4G20360	**−1.77**	−0.24	−0.38	−0.26	−0.36	−0.17	AtRABE1b/AtRab8D
AT4G36660	**−1.80**	−0.21	0.17	0.37	0.14	−0.12	similar to unknown protein
AT2G42570	**−1.86**	−0.81	0.19	−0.36	−0.25	−0.28	similar to unknown protein
AT3G02630	**−1.86**	0.20	−0.24	0.36	−0.01	0.33	acyl-(acyl-carrier-protein) desaturase
AT1G31230	**−1.87**	−0.11	0.04	0.01	−0.02	0.02	AK-HSDH
AT5G37310	**−1.88**	−0.45	0.10	−0.19	−0.10	−0.39	transporter
AT1G56190	**−1.89**	0.01	−0.21	−0.03	−0.04	−0.28	phosphoglycerate kinase, putative
AT3G11630	**−1.95**	−0.24	−0.29	−0.28	−0.14	−0.18	2-cys peroxiredoxin, chloroplast
AT3G54080	**−1.95**	−0.53	−0.07	−0.28	−0.13	−0.09	sugar binding
AT4G20980	**−1.98**	−0.58	0.02	−0.25	0.28	−0.39	eIF3d, putative
AT3G28700	**−2.03**	−0.18	−0.35	−0.09	−0.10	−0.33	similar to unknown protein
AT2G03780	**−2.03**	−0.37	−0.23	−0.10	0.07	−0.34	translin family protein
AT3G48730	**−2.06**	−0.21	−0.37	−0.09	0.03	−0.28	GSA2
AT3G51670	**−2.11**	−0.52	0.11	−0.26	0.05	−0.10	SEC14
AT1G23080	**−2.15**	−0.36	−0.30	0.32	0.39	−0.27	PIN7 (PIN-FORMED 7)
AT5G27990	**−2.16**	−0.31	−0.29	−0.17	0.38	−0.30	similar to unknown protein
AT2G36720	**−2.21**	−0.24	−0.38	−0.37	−0.09	−0.31	PHD finger transcription factor
AT3G53560	**−2.98**	0.00	−0.33	−0.21	0.16	0.25	chloroplast lumen protein

a, $\log_2$ expression ratio (treatment/control), the mean of two independent biological experiments.

Table 4. Genes affected only by ABA+SA and ABA+MeJA treatments (3 h).

Locus	Expression ratio[a]					Description
	SA	ABA+SA	ABA	ABA+MeJA	MeJA	
Up-regulated						
AT1G20180	0.22	**4.73**	0.38	**4.77**	0.03	similar to unknown protein
AT2G24762	0.01	**3.29**	0.32	**3.21**	0.01	ATGDU4
AT5G45630	0.21	**3.15**	0.09	**2.17**	−0.14	similar to unknown protein
AT4G18980	0.25	**3.11**	0.08	**3.35**	0.11	similar to unknown protein
AT1G79900	0.30	**2.73**	0.24	**3.03**	0.12	ATMBAC2/BAC2
AT3G10320	0.13	**2.26**	0.08	**2.80**	0.33	similar to unknown protein
AT1G63380	−0.18	**2.16**	−0.05	**1.78**	−0.05	short-chain dehydrogenase
AT4G32250	−0.26	**2.00**	−0.08	**2.41**	−0.17	protein kinase family protein
AT3G08870	0.01	**1.98**	−0.13	**1.34**	−0.33	lectin protein kinase, putative
AT5G50170	0.19	**1.85**	−0.30	**1.99**	−0.07	C2/GRAM domain-containing
AT1G73220	0.18	**1.78**	0.24	**2.08**	0.05	ATOCT1
AT5G64210	0.18	**1.75**	0.06	**1.34**	0.09	AOX2
AT3G11660	−0.02	**1.72**	−0.39	**2.10**	0.31	NHL1 (NDR1/HIN1-like 1)
AT2G31260	−0.13	**1.65**	0.01	**1.83**	−0.12	APG9 (AUTOPHAGY 9)
AT2G46030	0.21	**1.62**	0.06	**2.21**	0.02	UBC6
AT1G70610	0.14	**1.56**	−0.27	**1.83**	0.19	ATTAP1
AT5G65205	−0.09	**1.55**	0.02	**1.67**	−0.16	short-chain dehydrogenase
AT3G11840	−0.29	**1.53**	−0.09	**1.55**	−0.03	U-box domain-containing protein
AT5G24870	−0.22	**1.52**	−0.01	**1.96**	0.06	C3HC4-type RING finger protein
AT1G53670	−0.08	**1.46**	0.18	**1.30**	−0.12	MSRB1
AT3G61930	0.08	**1.44**	0.06	**3.26**	0.01	unknown protein
AT5G05930	−0.14	**1.38**	0.32	**1.43**	0.34	guanylyl cyclase-related (GC1)
AT5G03210	0.30	**1.36**	−0.26	**1.17**	−0.30	unknown protein
AT3G07940	0.32	**1.36**	0.21	**1.27**	0.29	zinc finger and C2 domain protein
AT5G44410	−0.18	**1.34**	−0.09	**1.42**	0.04	FAD-binding domain protein
Down-regulated						
AT3G53560	−0.33	**−1.66**	−0.16	**−2.98**	0.16	chloroplast lumen protein
AT2G36720	−0.38	**−1.21**	0.23	**−2.21**	−0.09	PHD finger transcription factor
AT5G27990	−0.29	**−1.70**	0.12	**−2.16**	0.38	similar to unknown protein
AT1G23080	−0.30	**−2.13**	−0.24	**−2.15**	0.39	PIN7 (PIN-FORMED 7)
AT3G51670	0.11	**−1.81**	−0.22	**−2.11**	0.05	SEC14 family protein
AT3G48730	−0.37	**−1.82**	−0.23	**−2.06**	0.03	GSA2
AT2G03780	−0.23	**−1.45**	−0.25	**−2.03**	0.07	translin family protein
AT3G54080	−0.07	**−2.19**	−0.40	**−1.95**	−0.13	sugar binding
AT3G11630	−0.29	**−1.62**	0.22	**−1.95**	−0.14	2-cys peroxiredoxin, chloroplast
AT5G37310	0.10	**−2.01**	−0.13	**−1.88**	−0.10	transporter
AT1G31230	0.04	**−1.42**	−0.23	**−1.87**	−0.02	AK-HSDH/AK-HSDH I
AT3G02630	−0.24	**−1.62**	−0.25	**−1.86**	−0.01	acyl-(acyl-carrier-protein) desaturase
AT4G36660	0.17	**−1.90**	0.04	**−1.80**	0.14	similar to unknown protein
AT4G20360	−0.38	**−1.51**	−0.08	**−1.77**	−0.36	AtRABE1b/AtRab8D
AT1G73940	−0.26	**−1.58**	−0.31	**−1.67**	0.19	similar to unknown protein
AT1G35680	−0.38	**−1.35**	0.07	**−1.67**	−0.03	50S ribosomal protein L21
AT5G60430	0.37	**−1.35**	0.14	**−1.65**	−0.24	antiporter/drug transporter
AT5G66680	−0.28	**−1.60**	0.31	**−1.65**	0.35	DGL1 (defective glycosylation 1)
AT5G23290	−0.07	**−1.32**	−0.33	**−1.65**	0.08	c-myc binding protein, putative
AT2G24170	−0.08	**−1.32**	−0.12	**−1.65**	−0.15	endomembrane protein 70
AT1G75330	−0.35	**−1.37**	−0.17	**−1.64**	0.05	OTC
AT1G06840	0.08	**−1.17**	−0.19	**−1.60**	−0.31	leu-rich repeat TM protein kinase
AT5G46160	−0.25	**−1.15**	−0.16	**−1.58**	0.13	ribosomal protein L14 family
AT1G06390	0.36	**−1.55**	0.11	**−1.56**	0.23	GSK1
AT3G50410	−0.34	**−1.88**	−0.20	**−1.48**	0.10	OBP1

a, $\log_2$ expression ratio (treatment/control), the mean of two independent biological experiments. b, p value <0.05. c, p value >0.05

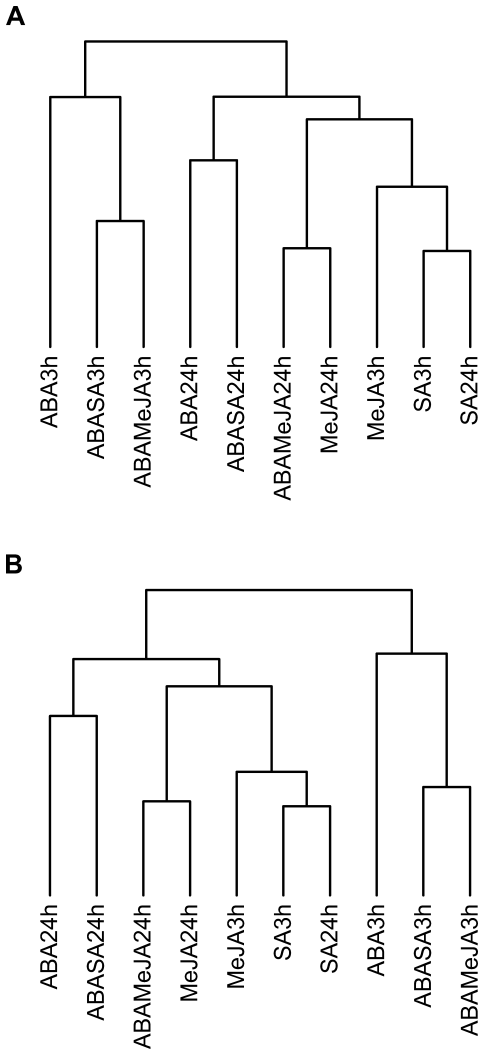

Figure 3. Hierarchical clustering analysis. Hierarchical clustering analysis of relative expression values for genes up- (A) and down-regulated (B) by hormone treatment. Genes with relative signal values ≥ 1 (2884 genes) or ≤ -1 (4274 genes) ($\log_2$ transformation) following any hormone treatment were analyzed. Clustering was performed by the agglomerative method within R software (hclust, agglomeration method "average").

Expression pattern of hormone-related genes

To explore the physiological implications of the antagonistic effects of ABA and SA or MeJA on gene expression, we examined the expression of genes involved in the biosynthesis and signaling of these hormones (Table 5). *NCED4* was significantly down-regulated by double hormone treatment compared to SA or MeJA alone. *CYP707A1* was negatively regulated by SA and MeJA, positively by ABA, and up-regulated by double hormone treatment. *CYP707A2* was up-regulated slightly more by ABA+SA than by ABA+MeJA. Most genes for ABA receptors were down-regulated by ABA, but *PYL4*, *PYL6*, and *PYL11* were more strongly down-regulated by double hormone treatments. In contrast, all genes encoding PP2Cs, which are negative regulators of ABA signaling, were more strongly up-regulated by double hormone treatment. Thus, in the presence of ABA, SA and MeJA enhance the feedback system of the ABA response. However, SA and MeJA alone did not have this effect, suggesting that SA and MeJA do not simply act antagonistically toward ABA.

The expression patterns of genes involved in SA biosynthesis and signaling factors were more complicated. An SA biosynthetic gene, *ICS1*/*EDS16*/*SID2*, and *NPR1*, which encodes an important transcription factor in the SA response, were down-regulated by ABA, consistent with an antagonistic relationship between ABA and SA. However, the effect of ABA+SA on these genes was unclear. The expression of *UGT74F2*, which encodes an SA glucose-conjugating enzyme (Song et al. 2009), was up-regulated by SA and further induced by ABA+SA. Presumably, this enzyme inactivates free excess SA; hence, ABA seems to have a role in reducing the SA response. By contrast, *SARD1*, which is involved in regulating the SA response, was up-regulated by ABA+SA (Wang et al. 2011), inconsistent with the antagonistic role of these hormones.

JA co-receptor genes were up-regulated by MeJA. Interestingly, most of these loci were down-regulated by ABA but up-regulated by ABA+MeJA. Because JA co-receptors negatively regulate the JA response, down-regulation of these genes should result in enhancement of the JA response, implying that ABA sensitizes the JA response in the absence of JA but reduces it in the presence of JA. Among JA co-receptor genes, *JAZ1*/*TIFY10A* was strongly up-regulated by ABA and further up-regulated by ABA+MeJA. This gene is uniquely regulated by auxin (Grunewald et al. 2009). It is possible that the locus is a key agent in the integration of plant hormone information. ABA+SA treatment up-regulated *JAZ1/TIFY10A* but SA alone did not, an outcome that confirms the integrative function of this gene.

Genes involved in cell cycle progression are strongly down-regulated by ABA+SA

Among 529 ABA and ABA+SA down-regulated genes [Figure S1D, section (b)], 32 genes whose expression was down-regulated 50% by ABA+SA treatment in comparison with ABA alone were selected. Interestingly, this set of genes was rich in loci predicted to have pivotal roles in cell cycle progression, especially in G2/M phase progression, nuclear division, and cytokinesis, including loci for several B-type cyclins, *CDKB2*, *aurora-like kinase2*, and others for motor protein-related proteins (Table 6) (Menges et al. 2002). The expression profiles of some of these loci were confirmed by quantitative real-time reverse transcription-PCR (RT-PCR) (Figure 4A). These 32 genes are active in the shoot and root apices where cells are actively dividing (Figure S2A).

Table 5. Response of genes involved in hormone regulation (3 h).

Locus	Expression ratio[a]					Description
	SA	ABASA	ABA	ABAMeJA	MeJA	
AT4G19170	−1.02	**−1.85**	−0.55	**−1.89**	−1.33	NCED4
AT4G19230	−0.89	**2.49**	**3.06**	**2.43**	−0.40	CYP707A1
AT2G29090	0.34	**1.63**	**1.22**	0.65	0.29	CYP707A2
AT5G46790	−0.45	−0.79	**−1.25**	**−1.80**	−0.39	PYL1
AT2G38310	−0.62	**−4.53**	**−3.75**	**−4.24**	0.36	PYL4
AT5G05440	−0.71	−2.36	**−2.75**	−2.19	−0.27	PYL5
AT2G40330	−1.36	**−1.67**	−1.37	−1.50	−0.15	PYL6
AT4G17870	−1.33	**−3.52**	−1.29	**−2.91**	0.98	PYL11
AT1G01360	−0.59	−0.58	**−1.76**	−0.94	−0.40	RCAR1
AT5G53160	0.02	−1.04	**−1.82**	−0.84	−0.15	RCAR3
AT5G66880	−0.06	−0.56	**−1.44**	−0.63	−0.44	SNRK2.3/SRK2I
AT4G33950	0.55	**2.10**	**1.71**	**2.11**	0.05	SnRK2.6/OST1/SRK2E
AT4G26080	0.54	**4.22**	**3.61**	**3.78**	0.38	ABI1
AT5G57050	0.87	**5.35**	**4.93**	**5.15**	−0.07	ABI2
AT1G72770	0.22	**2.79**	**1.82**	**2.64**	−0.04	HAB1
AT1G17550	−0.06	**2.85**	**1.58**	**2.74**	0.32	HAB2
AT5G51760	0.52	**1.05**	0.55	**1.13**	0.16	AHG1
AT3G11410	−0.16	**2.98**	**1.85**	**2.82**	−1.05	AHG3/PP2CA
AT5G59220	0.56	**6.43**	**5.58**	**5.87**	0.11	HAI1
AT1G07430	0.04	**6.88**	**6.05**	**6.18**	0.36	HAI2
AT2G29380	−0.17	**3.51**	**2.39**	**2.76**	0.13	HAI3
AT2G36270	−0.08	**1.45**	**1.53**	0.87	−0.21	ABI5
AT1G74710	−0.65	−1.22	**−2.20**	−1.00	−0.23	ICS1/EDS16/SID2
AT5G67160	**2.99**	**2.87**	−0.52	**1.38**	**1.87**	EPS1
AT1G73805	0.75	**1.57**	0.12	0.42	−0.16	SARD1
AT2G43820	**3.63**	**5.67**	−0.55	**2.64**	−0.19	UGT74F2
AT1G64280	0.33	−0.02	**−1.38**	−0.35	−0.20	NPR1
AT5G45110	**3.07**	**2.93**	0.24	0.60	−0.08	NPR3
AT4G19660	**1.79**	**1.06**	−0.57	−0.17	−0.32	NPR4
AT5G06950	−0.10	**−0.88**	−0.41	**−1.19**	−0.13	TGA2
AT2G06050	0.18	−0.62	**−1.08**	0.68	1.91	OPR3
AT2G39940	−0.10	−0.58	**−1.01**	−0.73	−0.61	COI1
AT1G19180	−0.25	**3.05**	**2.14**	**4.45**	**3.36**	JAZ1/TIFY10A
AT1G74950	−0.28	−0.06	**−1.44**	**1.04**	**2.81**	JAZ2/TIFY10B
AT3G17860	0.14	−0.51	**−1.74**	**1.13**	1.01	JAI3/JAZ3/TIFY6B
AT1G17380	0.28	0.51	0.28	**2.15**	**3.20**	JAZ5/TIFY11A
AT1G72450	0.01	0.85	**−2.37**	**2.43**	**2.13**	JAZ6/TIFY11B
AT1G30135	−0.07	−0.04	0.12	**1.32**	**2.73**	JAZ8/TIFY5A
AT1G70700	−0.07	−0.18	−0.36	0.90	**2.96**	JAZ9/TIFY7
AT5G13220	0.17	0.03	0.33	**3.65**	**5.75**	JAS1/JAZ10/TIFY9
AT5G20900	−0.25	**0.73**	−0.34	**1.57**	0.83	JAZ12/TIFY3B
AT4G17880	−1.10	**−2.15**	**−1.35**	**−2.11**	0.11	MYC4

a, $\log_2$ expression ratio (treatment/control, the mean of two independent biological experiments. Differential expressions supported with $p<0.05$ were highlighted in bold. Expressions of *NCED2*, *NCED3*, *NCED5*, *NCED6*, *NCED9*, *CYP707A3*, *CYP7074A*, *PYL2*, *PYL3*, *PYL7*, *SNRK2.2/SRK2D*, *ABF1*, *ABF3*, *ABF4*, *PAL1*, *EDS5*, *PAD4*, *MES1*, *MES9*, *MES4*, *UGT74F1*, *TGA5*, *TGA6*, *ATCAMBP25*, *CDR1*, *NSL1*, *SIZ1*, *CBP60G*, *LOX2*, *AOS*, *AOC1*, *AOC2*, *JAR1*, *JAZ4*, *JAZ7*, *MYC2*, *MYC3*, and *NINJA* were not significantly affected in these treatments.

Down-regulated expression of these genes has been found in transcriptomic analyses of diverse abiotic stressors (UV, night extension, and high osmotic stress), senescence inducing conditions, and in several mutant lines including *ang4-1/hub1-1* and in microRNA-resistant TCP4 transgenic plants (Figure S2B). HUB1 is a key regulator of the G2/M phase transition and of the endoreduplication cycle (Fleury et al. 2007) and the transcription factor TCP4 negatively regulates cell growth and the cell cycle (Sarvepalli and Nath 2011; Schommer et al. 2008). Together, these data suggest that the combination of ABA+SA reduces cell proliferation

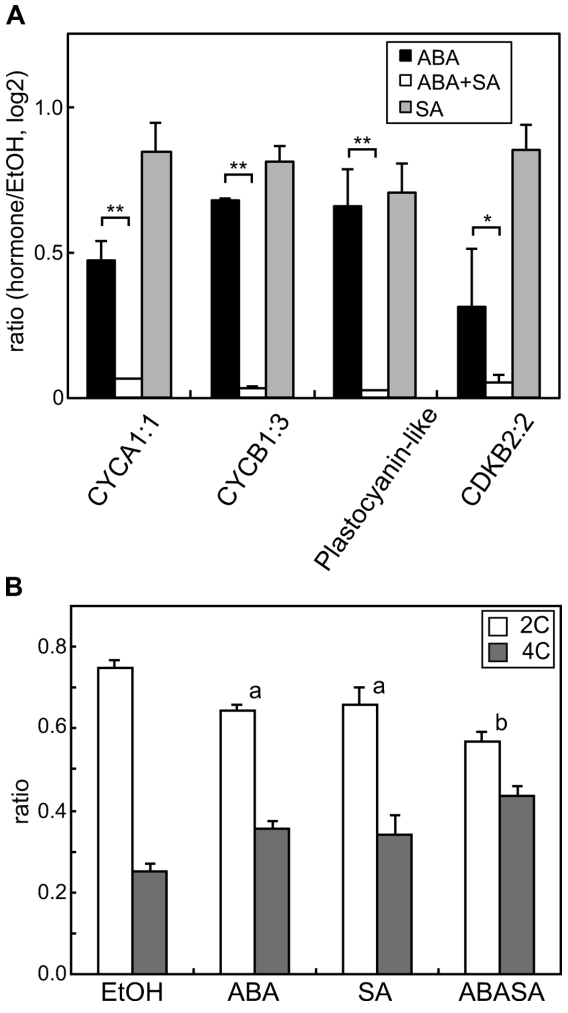

Figure 4. Effect of ABA+SA on cell cycle regulation. A, Expression profiles of 4 ABA+SA down-regulated genes (At1g44110, cyclin A1:1; At3g11520, cyclin B1:3; At4g31840, plastocyanin-like domain-containing protein; and At1g20930, cyclin dependent kinase 2:2) as analyzed by quantitative real-time RT-PCR. The expression values were normalized to the values for *ACTIN2* and are given as ratios of the control values (ethanol treatment). The values are means+SD (n≥3). * and ** indicate *P*-values (Student's *t*-test) of <0.2 and <0.05, respectively. (B) Cultured cells were treated with ABA, SA, or ABA+SA for 48 h, after which their nuclear DNA content was measured. The response ratios express the proportions of 4C and 2C DNA. The values are means+SD (n=3). Different lower case letters indicate significantly different means [Tukey's test following ANOVA ($P<0.05$)].

by down-regulating cell cycle-related genes. As shown in Table 6, most of these genes were also down-regulated significantly by MeJA treatment. MeJA down-regulates cell cycle-specific genes and inhibits cell cycle progression in cultured *Arabidopsis* cells (Pauwels et al. 2008). Consistent observations were made; thus, our findings are not an artifact.

To test our hypothesis, we analyzed the nuclear DNA content. Under normal growth conditions, 75% of cultured *Arabidopsis* T87 cells have a 2C DNA content and 25% have a 4C DNA content. Under our experimental conditions, cells with an 8C DNA content were detected very rarely. When treated with ABA or SA alone for 48 h, the ratio of cells with a 4C DNA content increased slightly while that of cells with a 2C DNA content decreased (Figure 4B). Treatment with ABA and SA together produced clearer effects: the ratio of cells with a 4C DNA content increased to 45%. These observations are consistent with our microarray data, and indicate that ABA+SA treatment induces a cell cycle arrest by reducing the expression of genes involved in the G2/M transition.

Discussion

ABA and SA are known to act antagonistically. Indeed, we found that the expression of some genes was reciprocally affected by ABA and SA (Table S2). However, more genes responded similarly to ABA and SA (Figure S1), suggesting that these hormones do not simply act in an antagonistic manner. In addition, more than 100 genes were responsive only to ABA and SA together (Table 2). Our data also suggest that SA enhanced the effect of ABA on ABA receptor and PP2C gene expression by accelerating the negative feedback effect. Thus, there is physiological cooperation between ABA and SA. This idea is consistent with our earlier work demonstrating that ABA+SA evoked a unique metabolic profile in plant cells (Okamoto et al. 2009). We also showed that ABA and SA cooperatively reduce the expression of genes involved in the G2/M transition (Figure 4A, Table 6). Presumably, the presence of ABA with SA acts as a distinct physiological signal that induces unique cellular responses. Plant cells challenged with pathogens transiently accumulate endogenous ABA and SA (Fan et al. 2009; de Torres-Zabala et al. 2007). Therefore, the simultaneous action of ABA and SA is a natural phenomenon. Although the physiological implications of ABA up-regulation during defense responses are controversial, our data strongly indicate that ABA plays important roles in plant defensive mechanisms.

The relationships between ABA and MeJA are more complicated than previously realized. In some cases, the hormones act antagonistically, while in other cases they act synergistically. Our transcriptomic data for ABA and MeJA are consistent with these previous observations. ABA and MeJA acted antagonistically for some genes, while for others they acted similarly (Table S2). We also identified genes that were regulated in cultured cells only by ABA+MeJA (Figure S1, Table 3). Interestingly, ABA+MeJA up-regulated JA receptor genes significantly while ABA treatment down-regulated many of them (Table 5). JA receptors negatively regulate the JA response by forming repressor complexes (Pauwels et al. 2010). Hence, the expression levels of JA receptor genes are among the determinants of JA sensitivity or

Table 6. Genes down-regulated further in ABA+SA 24 h-treatment

Locus[a]	Expression ratio[b]						Description[d]
	ABASA[c]	ABA[c]	SA	ABAMeJA	MeJA		
AT3G02120	−5.40	−4.18	−0.44	−3.78	−1.87		hydroxyproline-rich glycoprotein
AT4G31840	−5.37	−3.02	−0.46	−3.57	−2.21		plastocyanin-like
AT4G23800	−5.29	−4.22	−0.29	−4.41	−3.20		HMG1/2 family protein
AT1G08560	−4.80	−3.04	−0.30	−3.94	−2.37	*	SYP111 (syntaxin 111)
AT1G02730	−4.68	−3.55	−0.17	−3.54	−2.53	*	Cellulose synthase-like D5
AT4G33260	−4.66	−3.53	−0.30	−3.72	−2.73	*	CDC20.2; signal transducer
AT1G44110	−4.47	−3.37	−0.10	−3.74	−2.65	*	CYCA1;1
AT3G11520	−4.47	−3.52	0.03	−3.63	−2.14	*	CYCB1;3
AT5G01050	−4.43	−3.61	−1.21	−3.89	−1.51		laccase family protein
AT4G32830	−4.29	−3.35	−0.24	−3.48	−2.04	*	ATAUR1 (ATAURORA1)
AT2G25060	−4.19	−2.48	−0.20	−3.12	−2.15		plastocyanin-like
AT4G01730	−4.16	−3.35	−0.33	−3.21	−1.92		zinc finger (DHHC type)
AT1G03620	−4.11	−2.78	−1.02	−1.37	−0.65		ELMO1-related
AT1G53140	−4.03	−3.10	−0.21	−3.21	−2.32		dynamin family protein
AT1G20930	−4.01	−3.24	−0.14	−1.41	−0.68	*	CDKB2;2
AT2G26760	−3.98	−3.00	−0.43	−3.11	−1.74	*	CYCB1;4
AT5G49630	−3.94	−3.08	−1.30	−2.39	−0.10		AMINO ACID PERMEASE 6
AT1G76540	−3.72	−2.97	−0.27	−2.93	−1.69	*	CDKB2;1
AT2G27970	−3.70	−2.64	−0.21	−3.45	−1.94		CKS2 (CDK-SUBUNIT 2)
AT5G11510	−3.65	−2.80	−0.30	−3.08	−2.06		MYB3R-4
AT5G67270	−3.58	−2.54	−0.19	−2.89	−1.74	*	Microtuble end binding protein1
AT5G64100	−3.50	−2.63	−0.30	−0.89	−0.17		peroxidase, putative
AT4G28950	−3.41	−2.63	−0.16	−3.15	−1.79		ARAC7
AT1G16070	−3.34	−2.46	−0.04	−2.92	−1.73		TUBBY LIKE PROTEIN8
AT1G76310	−3.27	−2.50	−0.35	−2.83	−2.13	*	CYCB2;4
AT5G57220	−2.92	−1.96	−0.39	−1.60	−0.36		CYP81F2
AT4G39830	−2.73	−1.88	−1.38	−2.51	−2.44		L-ascorbate oxidase, putative
AT5G25090	−2.68	−1.93	−0.34	−2.62	−1.51		plastocyanin-like
AT2G25880	−2.50	−1.69	−0.40	−2.20	−2.01	*	ATAURORA2
AT2G17620	−2.15	−1.40	−0.16	−1.74	−1.22		CYCB2;1
AT1G12500	−2.14	−1.34	−0.78	−1.64	−0.72		phosphate translocator-related
AT5G27550	−2.10	−1.32	−0.40	−1.64	−1.58		kinesin like

a, genes without clear description were eliminated. b, $\log_2$ expression ratio (treatment/control), the mean of two independent biological experiments. c, p value <0.05. d, asterisks indicate the genes found in *ang4-1/hub1-1* down-regulated genes.

JA response levels. We suggest that the physiological signaling of these hormones changes when they are combined.

Multiple stimulus responses are of considerable current interest. The treatment of multi-factor diseases requires information on combinations and doses of drugs applied simultaneously. Protein level changes in response to multiple conflicting chemical stimuli have been investigated systematically in a human cancer cell line and in *E. coli* (Bollenbach and Kishony 2011; Geva-Zatorsky et al. 2010). Both model systems indicate that gene responses to multiple stimuli are linear superpositions of the responses to individual stimuli, suggesting a common process whereby cells are able to reduce complex multiple signals to the sum of the individual signals. We showed that the correlations between gene expression and the deduced values from the model were highly significant after 24 h of treatment with ABA+SA and ABA+MeJA (Figure 2). However, this was not the case following 3 h of treatment. Presumably, each gene reacts differently to paired hormone signals in the early response to paired hormones and the weights for each hormonal effect are different among genes. The fact that more genes were regulated only by double hormone treatment during our 3-h experiments supports this idea (Figure S1).

The linkage between the ABA+SA and ABA+MeJA treatments was striking. As shown in Figure 2, there was a clear close relation between these two treatments when applied for 3 h. There was significant overlap between loci that were up- or down-regulated only by ABA+SA and ABA+MeJA (Table 4). Most of them responded at the early stage (3 h) to double hormone treatment, suggesting that this was not a secondary effect. ABA+SA and ABA+JA probably have similar physiological effects. After challenging *Arabidopsis* with *Pseudomonas syringa*e, the cells produced SA, ABA, and JA sequentially (Fan et al. 2009); hence, paired hormone

signals may activate sets of defense-response genes *in planta*. According to a public transcriptome database, the genes in question respond to biotic and abiotic stresses. For example, BAC2/At1g79900, encoding an L-ornithine transporter involved in proline synthesis, is up-regulated by hyperosmotic stress, ABA treatment, and *P. syringae* infection (Toka et al. 2010). Such genes may function under the influence of multiple stressors.

Our detailed classification of hormone-regulated genes demonstrated that the coordinated effects of ABA and SA have physiological functions beyond defense. Strikingly, the loci down-regulated more by ABA+SA than by ABA and SA in 24-h experiments were rich in genes involved in cell cycle progression, especially the G2/M transition. The link between defense responses and cell cycle regulation has been reported previously (Ascencio-Ibáñez et al. 2008; Chandran et al. 2010). Wildermuth (2010) suggested that increases in gene number by endoreduplication might turn aside infectant attacks by increasing the number of target molecules for effectors (Wildermuth 2010). It is possible that during pathogen challenges, plants increase their levels of ABA and SA in order to induce this cell cycle phase change, which functions as a cell protection response. When cultured cells are treated with MeJA, similar cell cycle retardation occurs (Pauwels et al. 2008). We consistently observed down-regulation of cell cycle-related genes, such as cyclin and CDK2 loci, following MeJA treatment. However, MeJA had less effect than SA on the down-regulation of these genes by ABA; hence, MeJA and SA may well have different physiological functions in cell cycle regulation.

Previously, we reported the metabolic profile under hormone treatments (Okamoto et al. 2009). The data showed that the levels of primary metabolites, such as amino acids and sugars, were changed responding differently to ABA, SA and ABA+SA. However, the transcript levels of the genes in these metabolic pathways were not significantly affected. This observation is consistent with the idea that primary metabolite levels are mainly modulated by the post-transcriptional or post-translational regulation mechanisms of metabolic enzymes (Hirai et al. 2004).

We used cultured cells in our experiments in order to obtain data from homogeneous samples. Whole plants are the best material for physiological studies; however, to discern cellular responses to hormone signals, especially complex crosstalk, whole plants are difficult experimental systems because hormones penetrate different tissues differently, and different cells and tissues may have distinct hormone responses. In addition to cell homogeneity, the cell culture system has the advantage of being more readily treated with diverse chemicals that are difficult to apply to whole plants or tissues, and being easier to manipulate in terms of cell cycle progression. Using this system, we demonstrated that hormonal crosstalk is not simple as recognized before. However, it is possible that the responses of cultured cells do not reflect those *in planta*. Actually, we found that a few known SA-inducible genes were not activated in the cultured cells. This may well be a drawback of cultured cell systems. Therefore, the observed gene responses to hormones in cultured cells should be re-examined in adult plant tissues. Nevertheless our data offers clues to understand the basis of gene response to multiple hormones.

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