The Combined Effect of Drought Stress and Heat Shock on Gene Expression in Tobacco[1]

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In nature, plants encounter a combination of environmental conditions that may include stresses such as drought or heat shock. Although drought and heat shock have been extensively studied, little is known about how their combination affect plants. We used cDNA arrays, coupled with physiological measurements, to study the effect of drought and heat shock on tobacco (*Nicotiana tabacum*) plants. A combination of drought and heat shock resulted in the closure of stomata, suppression of photosynthesis, enhancement of respiration, and increased leaf temperature. Some transcripts induced during drought, e.g. those encoding dehydrin, catalase, and glycolate oxidase, and some transcripts induced during heat shock, e.g. thioredoxin peroxidase, and ascorbate peroxidase, were suppressed during a combination of drought and heat shock. In contrast, the expression of other transcripts, including alternative oxidase, glutathione peroxidase, phenylalanine ammonia lyase, pathogenesis-related proteins, a WRKY transcription factor, and an ethylene response transcriptional co-activator, was specifically induced during a combination of drought and heat shock. Photosynthetic genes were suppressed, whereas transcripts encoding some glycolysis and pentose phosphate pathway enzymes were induced, suggesting the utilization of sugars through these pathways during stress. Our results demonstrate that the response of plants to a combination of drought and heat shock, similar to the conditions in many natural environments, is different from the response of plants to each of these stresses applied individually, as typically tested in the laboratory. This response was also different from the response of plants to other stresses such as cold, salt, or pathogen attack. Therefore, improving stress tolerance of plants and crops may require a reevaluation, taking into account the effect of multiple stresses on plant metabolism and defense.

Under optimal conditions, cellular homeostasis is achieved by the coordinated action of many biochemical pathways. However, different pathways may have different molecular and biophysical properties, making them different in their dependence upon external conditions. Thus, during events of suboptimal conditions (stress), different pathways can be affected differently, and their coupling, which makes cellular homeostasis possible, is disrupted. This process is usually accompanied by the formation of reactive oxygen intermediates (ROIs) because of an increased flow of electrons from the disrupted pathways to the reduction of oxygen (Halliwell and Gutteridge, 1989; Noctor and Foyer, 1998; Asada, 1999; Dat et al., 2000; Mittler, 2002). One example for this process is the effect of heat shock on mitochondrial electron transfer. It was shown that during heat shock, membrane-bound complexes at the inner mitochondrial membrane are uncoupled or disrupted. Electrons from NADH produced by the soluble, and less temperature-sensitive, Krebs cycle enzymes are then channeled to the reduction of O_2 to ROI by different components of the uncoupled electron transport chain (Davidson and Schiestl, 2001).

To counter the effects of stress, plants undergo a process of stress acclimation. This process may require changes in the flow of metabolites through different pathways, the suppression of pathways that may be involved in the production of ROI during stress, and the induction of various defense genes such as heat shock proteins (HSPs) and ROI-scavenging enzymes (Vierling, 1991; Dat et al., 2000; Mittler, 2002).

The complexity of signaling events associated with the sensing of stress and the activation of defense and acclimation pathways is believed to involve ROI, calcium, calcium-regulated proteins, mitogen-activated protein kinase cascades, and cross talk between different transcription factors (Liu et al., 1998; Xiong et al., 1999; Bowler and Fluhr, 2000; Knight and Knight, 2001; Kovtun et al., 2000; Chen et al., 2002). Interestingly, different stress conditions such as drought and cold can result in the activation of similar stress response pathways (Seki et al., 2001; Chen et al., 2002). Thus, a high degree of overlap may exist between gene clusters activated by different stresses. This overlap may explain the well-documented phenomena of "cross tolerance," in which a particular stress can induce in plants resistance to a subsequent stress that is different from the initial one (Bowler and Fluhr, 2000).

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Although the study of abiotic stress response has advanced considerably in recent years, analyzing the effect of a single stress on plants can be very different from the conditions encountered by plants in the field in which a number of different stresses may occur simultaneously (Merquiol et al., 2001; Mittler et al., 2001). These can alter plant metabolism in a novel manner that may be different from that caused by each of the different stresses applied individually, and may require a new type of response that would not have been induced by each of the individual stresses.

To characterize some of the mechanisms involved in the response of plants to a combination of stresses, applied simultaneously, we studied the effect of drought and heat shock on tobacco (*Nicotiana tabacum*) plants. A combination of drought and heat shock can represent the conditions encountered by many plants and crops growing within arid and semiarid environments (Mittler et al., 2001); therefore, its understanding may be critical for the development of new strategies and tools to enhance stress tolerance via genetic manipulations.

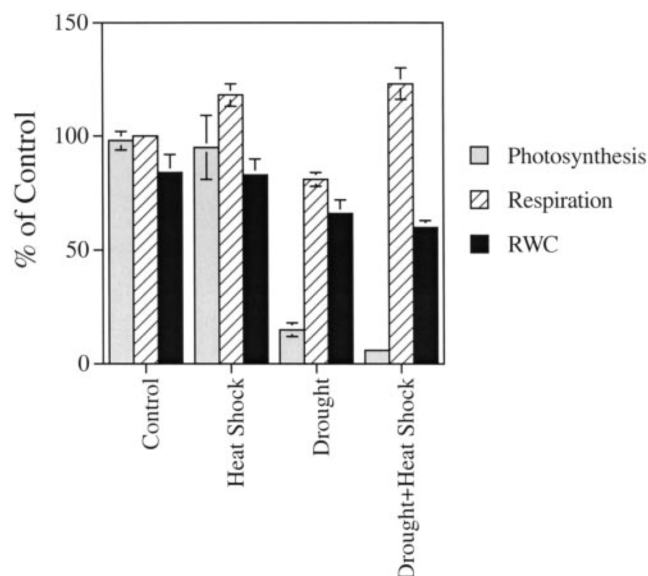

Figure 1. Measurements of photosynthesis and respiration in plants subjected to heat shock, drought stress, and a combination of heat shock and drought stress. Plants were subjected to stresses as described in "Materials and Methods," and photosynthetic activity and dark respiration were measured with an LI-6400 apparatus (LI-COR, Lincoln, NE). Photosynthetic activity is shown to be suppressed after drought stress or a combination of drought and heat shock, whereas respiration is enhanced after heat shock and a combination of drought and heat shock. A combination of drought and heat shock, therefore, is different from drought or heat shock by having a high rate of respiration and a low rate of photosynthetic activity. Results are presented as mean and SD of five individual measurements.

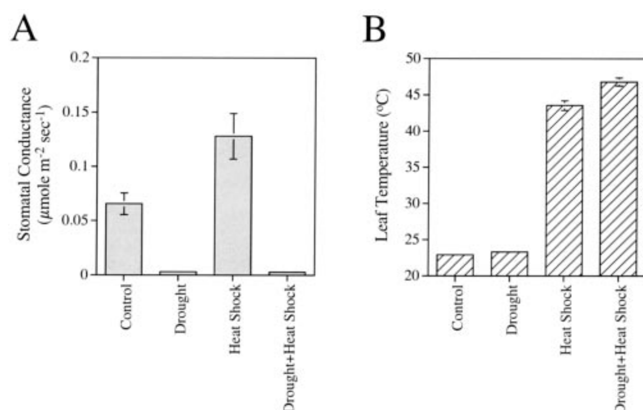

Figure 2. Stomatal conductance (A) and leaf temperature (B) of plants subjected to heat shock, drought stress, and a combination of heat shock and drought stress. Measurements were performed as described in "Materials and Methods." The temperature of leaves subjected to a combination of drought and heat shock is shown to be higher than that of plants subjected to heat shock in the absence of drought. This difference may result from the inability of plants, subjected to the stress combination, to cool their leaves by transpiration because their stomata are closed.

RESULTS

Physiological Characterization of Drought Stress, Heat Shock, and a Combination of Drought Stress and Heat Shock in Tobacco

To mimic the conditions encountered by plants during extended periods of drought, accompanied by brief exposures to heat shock (typically occurring between midday to late afternoon; Merquiol et al., 2001), we subjected tobacco plants to drought stress until they reached a relative water content (RWC) of 65% to 70%. Plants were then exposed to a heat shock treatment and sampled. As controls, we used well-watered plants (control), drought-stressed plants that were not subjected to heat shock (drought), and well-watered plants that were subjected to heat shock (heat shock). All plants were analyzed and sampled at the same time (after the heat shock treatment). Recovery tests indicated that plants subjected to a combination of drought stress and heat shock could recover within a few days upon watering and changing of temperature to 23°C (not shown). The conditions used in our study, therefore, were not lethal to plants.

As shown in Figure 1, drought stress resulted in the suppression of respiration and photosynthesis. In contrast, heat shock resulted in the enhancement of respiration, but did not significantly alter photosynthesis. Interestingly, the combination of drought stress and heat shock resulted in the suppression of photosynthesis, similar to drought stress, but the enhancement of respiration to levels that were comparable with those measured in plants after heat shock. Measurements of stomatal conductance, shown in Figure 2A, indicated that heat shock is accompanied by opening

of stomata, probably to enable the cooling of leaves via an enhanced transpiration stream. In contrast, stomata remained closed after drought or a combination of drought and heat shock, suggesting that plants subjected to a combination of drought and heat shock may be unable to cool their leaves by enhanced transpiration. Measurements of leaf temperature, shown in Figure 2B, revealed that the leaf temperature of plants subjected to a combination of drought and heat shock was higher by 2°C to 3°C compared with that of plants subjected to heat shock without drought. In addition, measurements of leaf transpiration confirmed that during heat shock transpiration is enhanced, whereas during a combination of drought and heat shock, transpiration is almost completely abolished (not shown). The results presented in Figures 1 and 2 suggest that a combination of drought and heat shock affects plants differently from drought or heat shock applied individually. The differences included changes in photosynthesis, respiration, stomatal conductance, and leaf temperature.

Molecular Characterization of Gene Expression during Drought Stress, Heat Shock, and a Combination of Drought Stress and Heat Shock in Tobacco

To examine the effect of drought and heat shock on gene expression in tobacco, we designed and used cDNA arrays composed of 170 cDNA clones encoding different defense and metabolic genes. These were spotted in duplicates on nylon filters and used to assay changes in the steady state level of their corresponding transcripts during drought, heat shock, and a combination of drought and heat shock. Identical filters were hybridized with radiolabeled cDNAs obtained from total RNA isolated from plants subjected to the different stresses. The overall pattern of gene expression detected by the filter arrays was different among control, drought stress, heat shock, and a combination of drought stress and heat shock (not shown). A summary of the changes in gene expression calculated as percent of control and averaged over five different experiments, each analyzed individually, is shown in Tables I through III. To compare the changes in expression during heat shock, drought stress, and a combination of drought stress and heat shock with other stresses, we subjected plants to salt stress, cold stress, PQ application, TMV infection, treatment with MJ, or to the expression of bO (Mittler et al., 1995). A summary of the changes in gene expression during these stresses is also shown in Tables I through III. As described previously, TMV infection and bO expression result in the activation of the hypersensitive response and the enhanced generation of ROI (Mittler et al., 1998). Because each of these additional stresses requires a different treatment, e.g. spraying with Tween 20 for PQ, mock

Table I. *Changes in the steady-state level of transcripts encoding heat shock proteins and ROI removal enzymes*

		Control		Heat		Drought		Drought+Heat													
Gene/Class		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Salt	SD	Cold	SD	PQ	SD	TMV	SD	bO	SD	MJ	SD
Heat Shock																					
	class II sHSP AI782242	**100**	66	**16770**	1089	**7516**	947	**47019**	1834	**203**	82	**67**	27	**nd**	nd	**nd**	nd	**nd**	nd	**54**	36
	chl sHSP AI776971	**100**	8	**11720**	777	**535**	201	**58688**	2859	**nd**	nd	**118**	20	**nd**	nd	**nd**	nd	**58**	100	**nd**	nd
	HSP18 (class I) M33899	**100**	20	**6493**	1447	**454**	234	**108046**	5516	**nd**	nd	**131**	83	**nd**	nd	**nd**	nd	**nd**	nd	**nd**	nd
	mit sHSP AI781457	**100**	65	**128**	115	**38**	7	**678**	446	**159**	12	**177**	11	**350**	240	**54**	8	**322**	15	**289**	36
	HSP100 AF083343	**100**	28	**130**	82	**205**	84	**928**	308	**46**	35	**10**	8	**216**	16	**42**	34	**60**	34	**26**	8
	HSP70 AF217458	**100**	40	**104**	36	**260**	27	**1072**	399	**57**	48	**33**	3	**220**	81	**95**	37	**86**	8	**22**	2
	HSP90 AW647639	**100**	4	**85**	63	**81**	29	**359**	199	**361**	68	**115**	25	**244**	10	**67**	12	**366**	302	**127**	34
	mit HSP60 AI483058	**100**	21	**122**	83	**128**	56	**480**	59	**175**	23	**65**	11	**209**	74	**83**	28	**132**	39	**101**	38
ROI-Removal	**Cu/ZnSOD** AI772848	**100**	21	**60**	32	**89**	17	**243**	76	**268**	131	**44**	6	**178**	14	**26**	3	**44**	20	**46**	23
	FeSOD AW621995	**100**	37	**82**	14	**122**	19	**170**	47	**183**	32	**94**	39	**164**	13	**74**	33	**233**	123	**137**	35
	ApxI U15933	**100**	17	**963**	491	**399**	111	**424**	139	**179**	65	**281**	16	**87**	49	**386**	41	**86**	51	**101**	45
	thy Apx AI776158	**100**	72	**235**	150	**176**	115	**103**	40	**nd**	nd	**nd**	nd	**156**	26	**31**	35	**127**	3	**221**	9
	str Apx AW093417	**100**	48	**36**	7	**61**	16	**92**	13	**282**	27	**91**	45	**142**	72	**77**	36	**348**	211	**128**	55
	Cat1 U93244	**100**	44	**67**	38	**182**	96	**56**	47	**102**	37	**15**	1	**239**	10	**8**	3	**90**	6	**99**	34
	Cat2 *Willekens et al.,*	**100**	56	**59**	23	**189**	23	**70**	23	**112**	80	**11**	3	**254**	32	**151**	26	**162**	67	**130**	5
	Cat3 *Willekens et al.,*	**100**	29	**187**	119	**239**	52	**162**	31	**93**	18	**30**	21	**213**	112	**262**	4	**192**	15	**80**	6
	GPX AW033598	**100**	22	**299**	127	**290**	72	**519**	109	**63**	19	**98**	64	**151**	22	**nd**	nd	**nd**	nd	**81**	21
	MDAR AI896762	**100**	36	**52**	44	**37**	14	**230**	106	**428**	110	**149**	96	**162**	76	**68**	7	**151**	68	**296**	16
	GR AW034391	**100**	28	**38**	26	**39**	10	**150**	14	**364**	9	**112**	64	**174**	53	**88**	37	**161**	58	**302**	7
	mit AOX S71335	**100**	11	**45**	14	**115**	51	**463**	125	**646**	80	**180**	2	**156**	38	**157**	22	**146**	74	**141**	45
	TPX AI490853	**100**	46	**362**	115	**153**	49	**167**	55	**69**	47	**144**	21	**107**	44	**nd**	nd	**nd**	nd	**43**	51
	GST AW039326	**100**	51	**158**	95	**133**	111	**313**	127	**203**	82	**67**	27	**nd**	nd	**nd**	nd	**nd**	nd	**54**	36

Changes in steady state level of transcripts are shown as % of control and standard deviation (SD) of 5 different experiments for the drought and heat shock experiments and 4 independent measurements of 2 different experiments for the other stresses (i.e., salt, cold, PQ, TMV, bO and MJ). Control 100% values are only shown on left for the drought and heat shock experiments. Control values with SD for the other experiments, i.e., salt, cold, PQ, TMV, bO and MJ, are not shown due to space limitations. Significant changes in gene expression ($P<0.05$) are indicated in red (induction) or blue (suppression). Genbank® accession numbers or references are indicated next to each of the clones. Abbreviations: AOX, alternative oxidase; APX, ascorbate peroxidase; CAT, catalase; chl, chloroplast; GPX, glutathione peroxidase; GST, glutathione-S-transferase; GR, glutathione reductase; HSP, heat shock protein; MDAR, monodehydroascorbate reductase; mit, mitochondria; MJ, methyl jasmonate; nd, not detected; PQ, paraquat; sHSP, small HSP; SOD, superoxide dismutase; TMV, tobacco mosaic virus; TPX, thioredoxin peroxidase.

Table II. *Changes in the steady-state level of transcripts encoding metabolic enzymes and proteins*

	Control		Heat		Drought		Drought+Heat													
Gene/Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Salt	SD	Cold	SD	PQ	SD	TMV	SD	bO	SD	MJ	SD
Photorespiration																				
Glycolate oxidase AW221447	**100**	41	**51**	23	**196**	34	**101**	51	**71**	36	**14**	1	**128**	6	**8**	3	**34**	18	**71**	32
Ser.hyd.met.tran. AI490845	**100**	18	**23**	9	**60**	16	**147**	91	**119**	14	**34**	3	**274**	29	**57**	8	**112**	8	**46**	13
Photosynthesis																				
PSII D2 AI483396	**100**	8	**68**	12	**68**	12	**73**	23	**64**	8	**22**	5	**80**	6	**52**	9	**54**	24	**59**	5
PSI P680 AW094640	**100**	17	**221**	74	**176**	49	**121**	62	**26**	12	**38**	17	**95**	52	**26**	1	**13**	8	**46**	2
PsaH AI491057	**100**	17	**56**	13	**33**	13	**45**	21	**52**	21	**18**	6	**91**	16	**2**	1	**21**	2	**38**	7
RbcS AI775905	**100**	37	**54**	31	**40**	17	**44**	50	**51**	32	**47**	26	**77**	22	**31**	17	**37**	17	**65**	12
RbcL AI776252	**100**	35	**158**	76	**200**	56	**191**	29	**152**	17	**49**	19	**109**	13	**30**	3	**107**	8	**172**	54
Cyt. B6F AW443034	**100**	27	**94**	37	**102**	40	**313**	140	**143**	48	**81**	23	**161**	51	**50**	8	**151**	34	**72**	8
Cab 1 AI482855	**100**	68	**66**	24	**80**	14	**36**	28	**46**	24	**13**	1	**58**	8	**4**	1	**15**	5	**22**	13
Thioredoxin h1 AW621673	**100**	25	**126**	82	**136**	63	**321**	86	**147**	64	**105**	32	**238**	164	**58**	22	**425**	243	**63**	36
Thioredoxin m4 AW625875	**100**	43	**152**	87	**133**	30	**133**	37	**87**	24	**35**	6	**158**	10	**11**	8	**42**	9	**68**	28
Respiration																				
Cyt. C oxidase AW625072	**100**	11	**175**	45	**135**	8	**153**	93	**178**	33	**65**	19	**325**	288	**44**	21	**46**	21	**511**	80
Cyt. B5 AW625941	**100**	38	**160**	111	**200**	49	**287**	42	**61**	23	**95**	26	**73**	63	**98**	29	**175**	64	**120**	7
Ubq cyt. C red. AW219610	**100**	12	**150**	130	**78**	22	**405**	253	**109**	46	**122**	49	**255**	144	**89**	12	**249**	46	**139**	18
ATP syntase AI490831	**100**	52	**36**	23	**42**	19	**185**	136	**65**	18	**41**	13	**274**	46	**192**	26	**398**	252	**280**	125
Sugar metabolism																				
G6PD AI491202	**100**	30	**63**	31	**255**	36	**299**	152	**94**	96	**97**	47	**279**	17	**556**	59	**145**	31	**97**	44
6-ph.gluc.lact. AW219886	**100**	41	**499**	114	**202**	58	**1728**	294	**236**	37	**283**	18	**263**	83	**nd**	nd	**180**	36	**127**	43
Ribose-5p-isom. AI777555	**100**	48	**121**	14	**120**	50	**95**	54	**nd**	nd	**62**	16	**nd**	nd	**nd**	nd	**nd**	nd	**nd**	nd
Trioseph. Iso. AW649951	**100**	16	**107**	21	**141**	12	**126**	25	**137**	24	**47**	12	**183**	149	**68**	31	**83**	15	**37**	14
Transketolase AW039152	**100**	30	**44**	11	**110**	26	**91**	39	**125**	10	**107**	12	**142**	119	**69**	39	**134**	26	**189**	13
Transaldolase AW621229	**100**	48	**70**	50	**95**	34	**369**	241	**295**	4	**236**	2	**126**	1	**170**	106	**164**	59	**189**	39
Aldolase AI483131	**100**	29	**66**	25	**150**	10	**156**	44	**60**	17	**45**	21	**82**	12	**5**	2	**23**	6	**33**	7
G3PD AI483087	**100**	31	**106**	42	**104**	28	**352**	59	**89**	48	**53**	36	**105**	102	**2**	42	**25**	22	**35**	29
Phosph.glyc. mut. AI777247	**100**	36	**96**	8	**142**	42	**104**	33	**nd**	nd	**nd**	nd	**nd**	nd	**10**	8	**132**	9	**311**	120
Invertase AI488187	**100**	21	**33**	10	**48**	8	**113**	49	**141**	47	**20**	3	**202**	11	**46**	30	**19**	15	**121**	83
Enolase AW649833	**100**	21	**64**	54	**59**	22	**261**	127	**285**	37	**109**	54	**191**	30	**68**	17	**80**	31	**189**	83
Pyruvate kinase AW625105	**100**	37	**54**	2	**155**	19	**344**	164	**46**	69	**267**	11	**83**	28	**60**	93	**466**	219	**192**	56
ADP-gl pyroph. AI776884	**100**	18	**30**	9	**72**	12	**109**	44	**63**	17	**36**	1	**nd**	nd	**7**	49	**32**	24	**56**	45

For table layout please see legends of Table 1. Abbreviations: 6-ph.gluc.lact., 6-phosphosgluconolactonase; ADP-gl pyroph., ADP-glucose pyrophosphorylase; Cab, chlorophyll a/b binding; Cyt, cytochrome; G3PD, glyceraldehyde-3-phosphate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; nd, not detected; Phosph.glyc.mut., phosphoglycerate mutase; PS, photosynthesis center; Red., reductase; Ribose-5p-isom, ribose-5-phosphate isomerase; Rbc, rubisco; Ser.hyd.met.tran, serine hydroxymethyltransferase; Triosph. Iso, triosephosphate isomerase; Ubq, ubiquinol.

infection for TMV, or growth in liquid culture for salt stress, an adequate control was designed for each treatment. These were critical because, as shown in Figures 3 and 4, and as described previously (Mittler and Zilinskas, 1992), different control treatments alter the expression of key genes such as APX and catalase. To confirm that these results, obtained with the cDNA arrays, adequately represent changes in steady-state transcript levels, we tested the expression of nine different cDNAs by RNA blots. These, shown in Figures 3 and 4, were found to be in good agreement with the results presented in Tables I through III (the results shown in Figs. 3 and 4 are from one experiment, whereas the results shown in Tables I through III are the average and SD of five different experiments, for the drought and heat experiments [$n = 5$], and the average of two different experiments each repeated twice for the other stresses [$n = 4$], including the experiments shown in Figs. 3 and 4). Because the leaf temperature of plants subjected to drought and heat shock was higher than that of plants subjected to heat shock in the absence of drought (Fig. 2), we performed additional experiments of heat shock at a higher temperature (i.e. 46°C); however, we did not find a significant difference between the induction of HSPs in tobacco plants subjected to heat shock at 46°C or heat shock at 44°C (not shown).

Table I summarizes results obtained for cDNAs encoding different HSPs, and different ROI removal enzymes. As shown in Table I, a number of HSPs were induced during a combination of drought and heat shock. These included cytosolic HSP90, HSP70, and HSP100, and sHSPs (cytosolic, mitochondrial, and chloroplastic). Overall, the induction of HSPs was higher in drought and heat shock compared with heat shock or drought. Analyzing the changes in ROI removal enzymes revealed interesting differences among the different stresses. During heat shock, cytosolic APX and thioredoxin peroxidase appeared to be dominant. In contrast, during drought stress, CAT and GPX appeared to be specifically induced. During a combination of drought and heat shock, however, AOX, GPX, glutathione reductase, CuZn-SOD, and glutathione-*S*-transferase were induced. Thus, the panel of transcripts encoding ROI-detoxifying enzymes induced during each of the different stresses appeared to be different, and ROI detoxification may occur via different routes during the different stresses. The induction of cytosolic APX during heat shock was in agreement with previous reports on the presence of a heat shock factor-binding sequence at the pro-

Table III. *Changes in the steady-state level of transcripts encoding general stress, ubiquitin, and "housekeeping" proteins*

	Control		Heat		Drought		Drought+Heat													
Gene/Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Salt	SD	Cold	SD	PQ	SD	TMV	SD	bO	SD	MJ	SD
General stress																				
DHN AW218656	100	22	205	132	11751	3091	2473	1383	566	146	58	10	445	57	575	131	824	123	155	51
Drougth DI19 AW094461	100	32	1733	38	545	263	3399	1796	nd	nd	210	125	119	101	94	14	146	95	nd	nd
Senescence 12 AW032486	100	23	81	61	73	35	328	168	93	20	64	15	198	74	123	36	340	214	78	22
Senescence 6 AW220559	100	30	70	44	50	16	162	60	130	64	79	22	164	4	57	27	224	17	118	46
Salt-induced AI490414	100	28	89	74	78	26	423	398	237	124	108	50	139	91	56	14	86	7	139	39
Cold-induced AW624918	100	35	132	61	173	49	488	206	189	37	250	7	265	7	132	36	204	45	53	11
Oxygenase AW224574	100	25	92	28	111	21	296	21	425	36	32	36	324	19	304	48	1306	461	640	295
Wound-Pin1 AI490668	100	28	48	26	89	51	317	199	94	7	69	30	134	8	112	58	605	92	46	3
Wound-Pin2 AI482620	100	53	54	39	43	23	348	213	415	155	136	90	239	154	34	8	816	23	180	84
Lox1 AI488229	100	33	305	99	225	12	666	260	nd	nd	78	29	144	62	3941	1100	292	13	401	89
PAL AI773222	100	41	38	19	118	58	660	354	64	16	64	13	168	23	365	185	425	40	123	16
CHS AW220759	100	41	34	13	53	27	300	279	69	15	84	40	157	73	92	10	318	17	70	17
PR-1 X12737	100	5	53	11	123	59	413	244	1174	326	95	28	2949	278	2872	1183	1583	23	177	48
PR-2 M60460	100	19	214	125	147	53	429	159	nd	nd	124	91	120	18	142	1	125	28	53	85
PR-3 Z11563	100	45	101	16	236	101	678	358	542	3	214	29	948	66	1579	546	1139	362	210	151
Pr degradation																				
UB AI491037	100	21	391	216	162	45	753	374	105	51	76	34	150	31	29	10	98	67	175	96
UB degradationAI488034	100	45	341	176	261	100	407	36	110	50	72	14	59	61	nd	nd	nd	nd	72	51
UB extension AW622522	100	44	432	253	291	45	408	266	144	11	57	21	135	11	98	31	144	16	60	20
Housekeeping																				
Tubulin α2 AW035578	100	31	117	14	105	44	72	15	79	81	104	31	174	27	195	126	nd	nd	80	42
Actin AI491056	100	25	167	61	316	133	213	45	75	1	51	20	238	54	100	11	58	14	128	29
Histone AW218844	100	37	366	109	735	240	439	111	45	21	45	25	120	49	53	27	52	22	112	34
EIF-5A Y63542	100	39	89	20	314	125	265	41	nd	nd	87	17	nd	nd	257	95	nd	nd	70	2

For table layout please see legends of Table 1. Abbreviations: CHS, chalcone synthase; DHN, dehydrin; DI, drought-induced; EIF, eukaryotic initiation factor; Lox, lipoxygenase; nd, not detected; PAL, phenylalanine ammonia lyase; Pin, protease inhibitor; PR, pathogen related; Prot., protein; UB, ubiquitin.

moter of *ApxI* (Mittler and Zilinskas, 1992; Storozhenko et al., 1998).

Changes in the steady-state transcript level of different metabolic genes are shown in Table II. As shown in Table II, many of the photosynthetic genes were suppressed during stress. Exceptions were transcripts encoding a PSI reaction center protein, the large subunit of Rubisco, and a subunit of cytochrome B6F. Because cyclic electron flow involves PSI and cytochrome B6F, it is possible that during stress some energy dissipation is obtained via this pathway. Glycolate oxidase, a key enzyme of the photorespiratory pathway induced during drought, was suppressed during a combination of drought and heat shock. In contrast to the suppression of photosynthetic genes, some transcripts encoding enzymes of the pentose phosphate pathway and glycolysis were induced during a combination of drought and heat shock. These included Glc-6-phosphate dehydrogenase and pyruvate kinase. The induction of these transcripts may suggest that during a combination of drought and heat shock, the flow of sugars through these pathways is enhanced, possibly for the production of reducing energy, such as NAD(P) H, in the absence of photosynthesis. In contrast to the suppression of transcripts involved in photosynthesis during a combination of drought and heat shock, transcripts encoding different components of the mitochondrial respiration pathway were not suppressed during a combination of drought stress and heat shock (Table II).

Table III summarizes changes in the expression pattern of different stress response genes. In contrast to drought or heat shock, a combination of drought and heat shock resulted in the induction of a number of different stress response transcripts. These included transcripts encoding PR proteins and PAL. In contrast to PR proteins that were not induced to the same extent as during TMV infection, PAL was induced to levels that were similar to or even higher than those found during pathogen infection. DHN, highly induced during drought stress, was only moderately induced during a combination of drought and heat shock (Table III; see also Fig. 3). In contrast, the induction of a different drought-induced protein (DI-19) was augmented by the combination of drought and heat shock. However, unlike DHN, this transcript was also induced during heat shock. The induction of transcripts encoding different components of the UB protein degradation pathway was also elevated during a combination of drought and heat shock. The induction of the different stress and pathogen response transcripts during a combination of drought and heat shock suggests that this combination may have activated a signal transduction pathway that is also activated during wounding or pathogen infection. This activation might have resulted from the combined synthesis of different plant hormones such as abscisic acid, ethylene, and MJ. The expression of lipoxygenase, involved in jasmonic acid synthesis, was elevated during drought and heat shock.

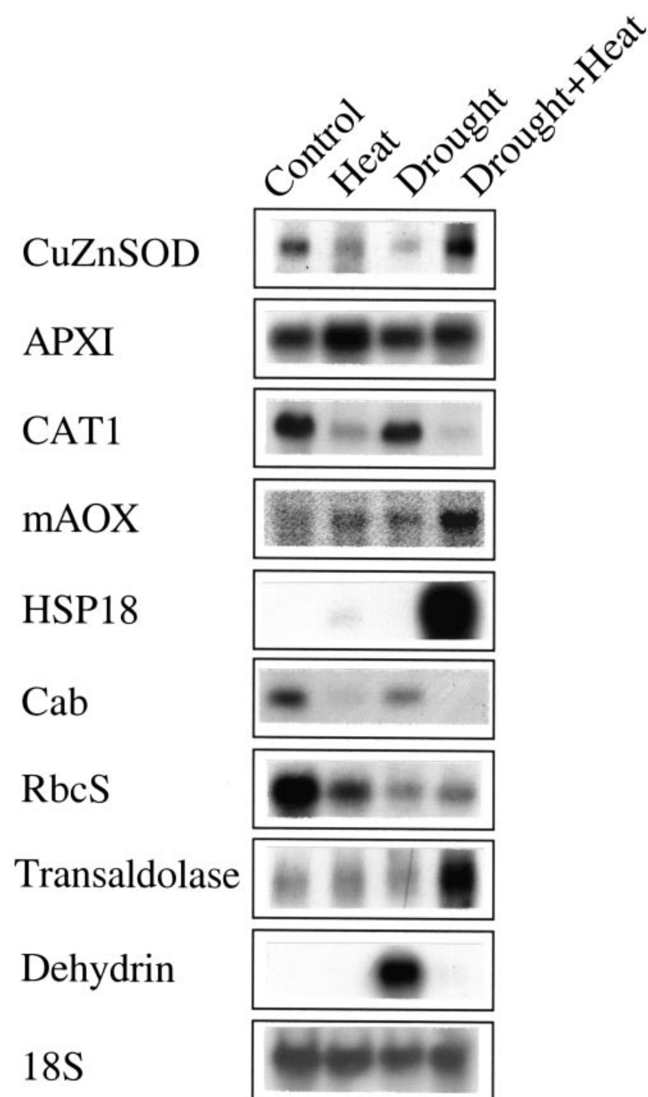

Figure 3. Changes in the steady-state level of transcripts encoding stress response and metabolic proteins and enzymes during a combination of drought and heat shock. RNA gel blots were used to assay the steady-state level of selected transcripts during a combination of drought and heat shock. Many of the transcripts shown in this figure have a distinct expression pattern during a combination of drought and heat shock. RNA isolation, blots, and analysis are described in "Materials and Methods."

Expression of Stress Response Transcripts with Homology to Transcripts Isolated from the Desert Plant *Retama raetam* during a Combination of Drought Stress and Heat Shock in Tobacco

We recently cloned, by a subtraction cDNA cloning method, a number of stress response cDNAs induced in the desert plant *R. raetam* in response to a combination of different naturally occurring stresses, of which drought and heat shock appear to be the most prominent (Pnueli et al., 2002). To test whether homologs of these transcripts are also involved in the response of laboratory-grown plants to a combination of stresses, we studied their expression in tobacco plants subjected to drought, heat shock, and a combination of drought and heat shock.

As shown in Figure 5, the expression of two transcripts with a high degree of homology to transcripts induced in the desert plant, i.e. those encoding a WRKY transcription factor, and an ethylene response transcriptional co-activator (ERTCA), was specifically induced during a combination of drought and heat shock in tobacco. The specific induction of these transcription factor homologs during a combination of drought and heat shock may suggest that this combination is accompanied by the activation of a unique genetic program different from the programs activated in plants during drought or heat shock. The expression of another transcript, i.e. a homolog PR-10, induced in the desert plant (Pnueli et al., 2002), was also induced during a combination of drought and heat shock. However, this transcript was also induced during heat shock in the absence of drought. In contrast, a homolog of a novel transcript corresponding to the Arabidopsis gene AC007508.2, induced in the desert plant (Pnueli et al., 2002), was not specifically induced during a combination of drought and heat shock in tobacco (Fig. 5).

DISCUSSION

We performed an initial characterization of the response of tobacco plants to a combination of drought stress and heat shock. Our results strongly suggest that the effect of this combination on plants is very different from that of drought or heat shock applied individually. Because in the field or in nature plants are often subjected to a combination of stresses such as drought and heat shock, studying the response of plants to a combination of different stresses may be critical to our understanding of stress toler-

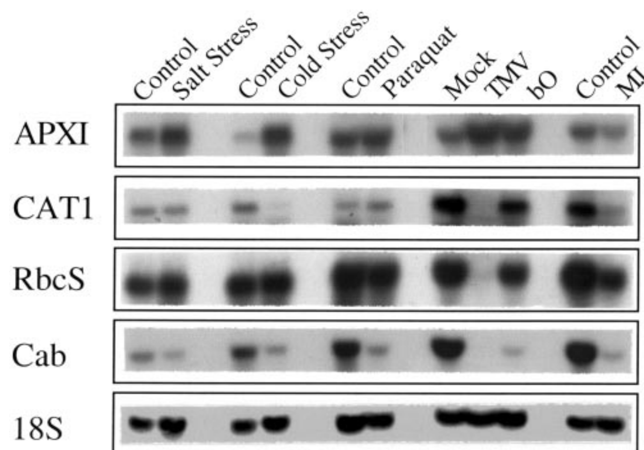

Figure 4. Changes in the steady-state level of transcripts encoding stress response and metabolic proteins and enzymes after different environmental stresses. RNA gel blots were used to assay the steady-state level of selected transcripts during different stresses. RNA isolation, blots, and analysis are described in "Materials and Methods."

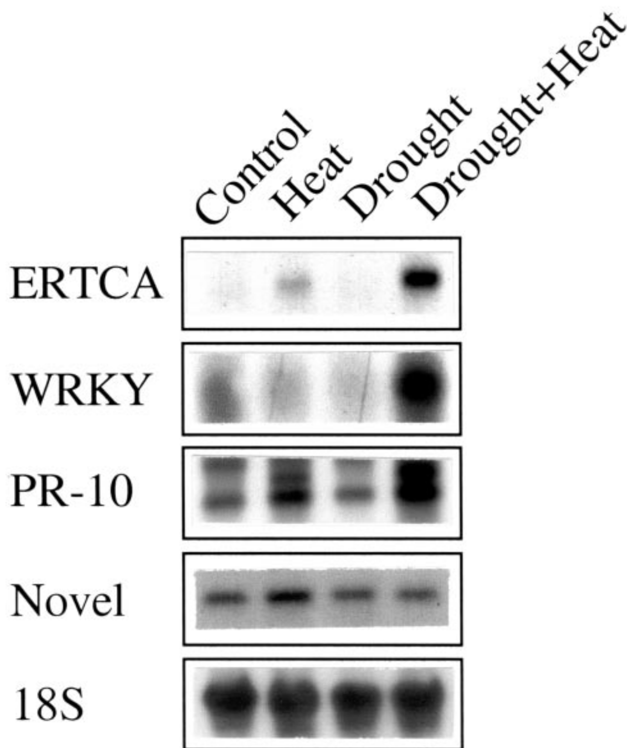

Figure 5. Expression of transcripts with homology to stress response cDNAs isolated from the desert plant *R. raetam*. RNA gel blots were used to study the expression of different transcripts that hybridized to cDNAs isolated from the desert plant *R. raetam* subjected to a combination of drought and heat shock in its natural environment. Hybridizations were performed at a high stringency (60°C) using full-length *R. raetam* cDNA clones as described in "Materials and Methods."

ance in plants. Thus, stress combinations such as drought and cold, heat shock and high light, or drought and heat shock should be studied before a successful manipulation of plant metabolism can be achieved, to artificially enhance stress tolerance. Future studies using full-scale genome arrays conducted on Arabidopsis plants subjected to similar stress combinations may reveal key regulators of gene clusters activated during a combination of stresses. The identification of two transcripts encoding homologs of proteins involved in the transcriptional regulation of gene expression, i.e. WRKY and ERTCA, specifically induced during a combination of drought and heat shock (Fig. 5), supports the presence of key regulators involved in this response. The finding that a combination of drought and heat shock results in the activation of wound and pathogen response pathways, not activated by each of these stresses applied individually, can also be viewed as an evidence for the induction of a unique genetic program upon stress combination. Our results, therefore, may provide an entry point and a reference to future analysis of gene expression during a combination of stresses. In addition, our results can suggest possible targets for the enhancement of stress tolerance in crops by genetic engineering. Thus, it may be possible to enhance the tolerance of plants to multiple stresses by manipulating the expression of different enzymes of the pentose phosphate pathway, AOX, GPX, and/or homologs of the transcription factors identified by our study (i.e. WRKY and ERTCA).

A number of new findings were uncovered by our analysis. For example, a role for mitochondrial AOX and GPX in the protection of cells from ROI-related damage during a combination of stresses can be suggested. In addition, the finding that the expression of DHN is suppressed during a combination of drought and heat shock may suggest that during this combination, HSPs can replace the stabilizing function of DHN, and it is no longer required for drought-related cellular protection. The source of NAD(P) H used for the removal of ROI during stress is mostly unknown. Our results suggest that the reduction of NAD(P)+ to NAD(P) H during stress, in the absence of photosynthesis, may occur via the pentose phosphate pathway. This suggestion is supported by a number of studies in animal cells and yeast (*Saccharomyces cerevisiae*), linking the pentose phosphate pathway to the removal of ROI during normal metabolism and stress (Pandolfi et al., 1995; Juhnke et al., 1996), and by our recent findings that plants with suppressed expression of APX and CAT have enhanced expression of transcripts encoding enzymes of the pentose phosphate pathway (Rizhsky et al., 2002). The expression of transcripts encoding enzymes of the pentose phosphate pathway was also elevated during other stresses such as PQ and salt (Table II).

Drought stress and heat shock may affect plant metabolism in a different manner when applied individually. However, it is not entirely clear how they affect plant metabolism when occurring simultaneously. Our analysis suggests that the mitochondria may be critical during a combination of drought and heat shock. During this combination photosynthesis is suppressed, whereas respiration is enhanced (Fig. 1). In addition, the expression of photosynthetic genes is suppressed, whereas the expression of genes involved in respiration is unchanged or induced (Table II). Moreover, the expression of mitochondrial AOX, implicated in the defense of plants from mitochondria-generated ROI during stress (Maxwell et al., 1999), is specifically elevated during a combination of drought and heat shock (Table I; Fig. 3). However, the exact role of the mitochondria, aside from energy supply in the absence of photosynthesis, is unknown.

The response of plants to a combination of drought and heat shock is composed of suppression of photosynthesis, enhancement of respiration, induction of a large number of defense genes, including genes induced during pathogen defense, and changes in genes involved in sugar metabolism. The overall bal-

ance between the expression of transcripts encoding different ROI removal enzymes and HSPs is also altered during a combination of drought and heat shock. These changes strongly suggest that the combination of drought and heat shock results in the activation of a unique genetic program that is different from that activated during drought or heat shock. Comparing the expression pattern of the different transcripts shown in Tables I through III between the combination of drought and heat shock and other stresses, such as cold, salt, PQ, or pathogen attack, suggests that the response of plants to the stress combination is also different from the response of plants to these stresses.

Drought and heat shock combination resulted in the induction of at least one senescence-associated transcript (SAG12; Table III). An overlap in the activation of at least 28 different transcription factors was recently reported between senescence and environmental stresses such as cold, salt, and pathogen attack (Chen et al., 2002). Therefore, it is possible that some overlap may also exist between senescence and a combination of drought and heat shock. Interestingly, the study of Chen et al. (2002), although very comprehensive, could not assign a function to a specific WRKY protein, identified as the Arabidopsis homolog of NtWRKY4, also a homolog of the *R. raetam* WRKY used for the hybridizations shown in Figure 5. From our results (Pnueli et al., 2002; Fig. 5), it is possible that this WRKY is involved in the response of plants to a combination of stresses such as drought and heat shock, or drought and cold stress.

MATERIALS AND METHODS

Growth Conditions and Physiological Measurements

Growth of tobacco (*Nicotiana tabacum* cv Xanthi-nc NN) plants and experiments were conducted under controlled environmental conditions at 23°C or 44°C. Plants were individually potted in equal amounts of Pro-Mix, and watered with 0.5× Hoagland solution. Continuous illumination was provided by cool-white fluorescent lamps (150 μmol m^{-2} s^{-11}). Photosynthetic activity, dark respiration, leaf temperature, and stomatal conductance were measured with a LI-COR LI-6400 apparatus using the following measuring cell (6 cm^2) parameters: 23°C or 44°C, 150 μmol photons m^{-2} s^{-1}, and an air flow of 300 μL s^{-1}, as previously described (Mittler et al., 2001). RWC was determined as described by Mittler and Zilinskas (1994).

Stress Treatments

Heat shock was applied by raising the temperature in the growth chamber to 37°C for 1 h, followed by another increase to 44°C for 6 h. Drought stress was imposed by withdrawing water from plants until they reached a RWC of 65% to 70% (typically 6–7 d). A combination of drought and heat shock was performed by subjecting drought-stressed plants (RWC of 65%–70%) to the heat hock treatment. All plants, i.e. drought-stressed plants, well-watered plants subjected to heat shock, drought- and heat-shocked plants, and control well-watered plants kept at 23°C were sampled at the same time for analysis. Cold stress was imposed by changing the temperature in the growth chamber to 4°C for 48 h. Control plants were kept at 23°C. Mock TMV infection plants expressing the bO gene and treatment of plants with MJ were performed as described previously (Mittler et al., 1998). PQ treatment was performed as described by Mittler and Zilinskas (1992). Salt stress was induced by subjecting 7-d-old tobacco seedlings, grown in culture in a medium containing 0.5× Hoagland, to 250 mM NaCl for 3 d. Control seedlings were grown in the same culture media without NaCl. For all stresses, control and stressed tissue were sampled at the same time.

RNA Isolation and RNA Gel Blots

Total RNA was isolated as previously described (Mittler et al., 1998) and subjected to RNA gel-blot analysis (Mittler and Zilinskas, 1992). A probe for 18S rRNA was used to ensure equal loading of RNA. Hybridization conditions were as follows: 0.25 M Na_2HPO_4, 1 mM EDTA, 7% (w/v) SDS, and 1% (w/v) casein (pH 7.4) at 60°C to 65°C, overnight, and washes were at 1× SSC and 0.1× SSC in the presence of 0.1% (w/v) SDS.

Filter Array Hybridization

Clones for the production of filter arrays were ordered from the tomato (*Lycopersicon esculentum*) expressed sequence tag library at Clemson University (SC), or obtained from the laboratories of Drs. Dirk Inzé (University of Gent, Belgium), Barbara A. Zilinskas (Rutgers University, NJ), Pierre Goloubinoff (Hebrew University, Jerusalem, Israel), and Gadi Schuster (Technion, Haifa, Israel). Filter cDNA arrays were prepared from the clones by spotting PCR products in duplicates on nylon membranes at the Hadassah Medical School DNA Facility of the Hebrew University. Filters were hybridized with radiolabeled cDNAs prepared from total RNA isolated from the different plants using oligo-dT and Superscript reverse transcriptase (Life Technologies/Gibco-BRL, Cleveland) as suggested by the manufacturer. Hybridization conditions were as follows: 57°C, 5× SSC, 5× Denhart, 0.5% (w/v) SDS, and 100 μg mL^{-1} salmon sperm DNA, overnight. Washing conditions were as follows: 57°C, 2× SSC, and 0.1% (w/v) SDS for 20 min, followed by 0.2× SSC and 0.1% (w/v) SDS, 57°C, for 20 min. After hybridization and washes, the signals were assayed with a phosphor imager (BAS1000, Fuji Photo Film, Tokyo) and analyzed with TINA software (Raytest, Pittsburgh). A number of control "housekeeping" genes, animal-specific genes (as negative controls), and empty spots (for background) were also spotted on the membrane. These were used to normalize the intensity of signals between the different filters and calculate the changes in gene expression presented in Tables I through III. When pertinent, the expression level of specific genes was verified by RNA blots.

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