Enhanced Tolerance to Environmental Stress in Transgenic Plants Expressing the Transcriptional Coactivator Multiprotein Bridging Factor 1c[1][w]

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Abiotic stresses cause extensive losses to agricultural production worldwide. Acclimation of plants to abiotic conditions such as drought, salinity, or heat is mediated by a complex network of transcription factors and other regulatory genes that control multiple defense enzymes, proteins, and pathways. Associated with the activity of different transcription factors are transcriptional coactivators that enhance their binding to the basal transcription machinery. Although the importance of stress-response transcription factors was demonstrated in transgenic plants, little is known about the function of transcriptional coactivators associated with abiotic stresses. Here, we report that constitutive expression of the stress-response transcriptional coactivator multiprotein bridging factor 1c (MBF1c) in Arabidopsis (*Arabidopsis thaliana*) enhances the tolerance of transgenic plants to bacterial infection, heat, and osmotic stress. Moreover, the enhanced tolerance of transgenic plants to osmotic and heat stress was maintained even when these two stresses were combined. The expression of MBF1c in transgenic plants augmented the accumulation of a number of defense transcripts in response to heat stress. Transcriptome profiling and inhibitor studies suggest that MBF1c expression enhances the tolerance of transgenic plants to heat and osmotic stress by partially activating, or perturbing, the ethylene-response signal transduction pathway. Present findings suggest that MBF1 proteins could be used to enhance the tolerance of plants to different abiotic stresses.

Abiotic stress conditions cause extensive losses to agricultural production worldwide (Boyer, 1982; Bray et al., 2000; Hoerling and Kumar, 2003; Rosegrant and Cline, 2003; Peters et al., 2004). Key to the tolerance of plants to abiotic stresses is a complex network of transcription factors and other regulatory genes that control multiple defense enzymes, proteins, and pathways (Bray et al., 2000; Cushman and Bohnert, 2000). Although the important role of many stress-response transcription factors was demonstrated in transgenic plants subjected to abiotic stresses (e.g. Kasuga et al., 1999; Mishra et al., 2002; Maruyama et al., 2004; Vogel et al., 2005), little is known about the function of other components of the plant transcriptional machinery during stress.

Transcriptional coactivators play a crucial role in eukaryotic gene expression by communicating between transcription factors and/or other regulatory components and the basal transcription machinery. They are divided into two classes: transcriptional coactivators that recruit or possess enzymatic activities that modify chromatin structure (e.g. acetylation of histone) and transcriptional coactivators that recruit the general transcriptional machinery to a promoter where a transcription factor(s) is bound (Näär et al., 2001). Multiprotein bridging factor 1 (MBF1) is a highly conserved transcriptional coactivator involved in the regulation of diverse processes such as endothelial cell differentiation, hormone-regulated lipid metabolism, central nervous system development, and His metabolism (Takemaru et al., 1997, 1998; Brendel et al., 2002; Liu et al., 2003). MBF1 proteins from different organisms interact with transcription factors such as c-Jun, GCN4, and ATF1, or with different nuclear receptors, and link them with the TATA-binding protein (Takemaru et al., 1997, 1998; Brendel et al., 2002; Busk et al., 2003; Liu et al., 2003). The flowering plant Arabidopsis (*Arabidopsis thaliana*) contains three different genes encoding MBF1. Functional assays demonstrate that all three Arabidopsis genes can complement MBF1 deficiency in yeast (Tsuda et al., 2004). MBF1a (At2g42680) and MBF1b (At3g58680) are developmentally regulated (Tsuda and Yamazaki, 2004). In contrast, the steady-state level of transcripts encoding MBF1c (At3g24500) is specifically elevated in Arabidopsis in response to pathogen infection, salinity, drought, heat, hydrogen peroxide, and application of the plant hormones abscisic acid or salicylic acid (Rizhsky et al., 2004b; Tsuda and Yamazaki, 2004; a search of 1,800

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ATH1 chips at https://www.genevestigator.ethz.ch/). The level of transcripts encoding MBF1c or its orthologs is also elevated in response to a combination of drought and heat in Arabidopsis, tobacco (*Nicotiana tabacum*), and the desert legume *Retama raetam* (Pnueli et al., 2002; Rizhsky et al., 2002, 2004b). However, the relative contribution of MBF1c to biotic and abiotic stress tolerance is unknown.

Here, we report that constitutive expression of the transcriptional coactivator MBF1c in Arabidopsis enhances the tolerance of transgenic plants to bacterial infection, salinity, heat, and osmotic stress, and that the enhanced tolerance of transgenic plants to heat and osmotic stress is maintained even when these two stresses are combined. We further show that MBF1 expression enhances the tolerance of transgenic plants to heat and osmotic stress by partially activating, or perturbing, the ethylene-response signal transduction pathway. MBF1 proteins could, therefore, be used to enhance the tolerance of plants to different abiotic stresses.

RESULTS

Production and Characterization of Transgenic Arabidopsis Plants Expressing MBF1c

To test the function of MBF1c in Arabidopsis, we generated transgenic plants that constitutively express MBF1c under the control of the 35S cauliflower mosaic virus promoter and subjected them to biotic or abiotic stresses. Transcripts encoding MBF1c accumulated in transgenic plants grown under controlled conditions to levels that were comparable to or higher than those detected in wild-type plants subjected to heat stress, drought, or a combination of heat stress and drought (Fig. 1A; Supplemental Fig. 1; Rizhsky et al., 2004b). In contrast, the level of transcripts encoding MBF1a or MBF1b did not significantly accumulate in wild-type plants subjected to heat stress, drought, or a combination of heat stress and drought (Fig. 1A). The expression of MBF1a or MBF1b was also not altered in transgenic plants expressing MBF1c (MBF1c-OE; Fig. 1A). Transgenic plants expressing MBF1c appeared similar in their growth and development to wild-type plants. However, as shown in Figure 1B, transgenic plants expressing MBF1c were 20% larger than control plants and produced more seeds. In addition, compared to wild-type plants, transgenic plants expressing MBF1c bolted 2 to 3 d earlier. As shown in Figure 2A, the basal thermotolerance of 4- to 5-d-old MBF1c-expressing seedlings was higher than that of wild-type seedlings of similar age and size (measured as survival rate following a 2-h 45°C heat stress). As shown in Figure 2B, 2-week-old MBF1c-expressing plants were more resistant than wild-type plants to bacterial growth (measured as suppressed in planta bacterial population size following inoculation of plants with 50 colony forming units [cfu] cm^{-2}). In addition, when grown under high-light conditions (1,000 μmol m^{-2} s^{-1} for 48 h), 2-week-old MBF1c-expressing plants accumulated a higher level of anthocyanin than wild type (Fig. 2C). In contrast, the tolerance of 4- to 5-d-old transgenic seedlings expressing MBF1c to cold stress was similar to that of wild-type seedlings (data not shown).

To study the tolerance of MBF1c-expressing plants to osmotic or salinity stress, we subjected 4- to 5-d-old wild-type and MBF1c-expressing seedlings to these stresses on agar plates and measured their root growth. In addition, to examine whether the tolerance of transgenic plants to these stresses is maintained when they are combined with heat stress, we subjected 4- to 5-d-old seedlings to a combination of heat and osmotic stress and to a combination of heat and salinity stress. As shown in Figure 3A, MBF1c-expressing plants were more tolerant than wild-type plants to heat or osmotic stress. Furthermore, the tolerance of transgenic plants to heat or osmotic stress was maintained even when these two stresses were combined (Fig. 3A). MBF1c-expressing plants were more tolerant than wild-type plants to a low level of salinity stress (i.e. 50 mM; data not shown). In contrast to the results obtained with heat and osmotic stress combination (Fig. 3A), the tolerance of MBF1c-expressing plants to salinity stress was not maintained when salinity stress was combined with heat stress (data not shown). As shown in Figure 3, B and C, the tolerance of transgenic plants expressing MBF1c to a combination of osmotic and heat stress was also evident from survival assays in which transgenic plants were compared to wild-type plants in survival assays similar to those shown in Figure 2A. In contrast, the survival rate of transgenic seedlings expressing MBF1c to a combination of salinity and heat stress was similar to that of wild type (data not shown).

Transcriptional Profiling of MBF1c-Expressing Plants

To test whether constitutive expression of MBF1c in transgenic plants results in the accumulation of different stress-response transcripts under controlled conditions, similar to the effect of constitutively expressing a defense-response transcription factor (e.g. Maruyama et al., 2004; Vogel et al., 2005), we performed transcriptional profiling of wild-type and transgenic plants. Table I summarizes all transcripts with a known or putative function elevated in MBF1c-expressing plants under controlled conditions. As shown in Table I, the constitutive expression of MBF1c resulted in the accumulation of transcripts encoding a number of stress-response transcription factors and signal transduction genes. These include WRKY and CBF-like transcription factors, MAPK3/11, and calcium-binding proteins. Ethylene was shown to play an important role in the defense response of plants against heat stress (Larkindale et al., 2005). In this respect it was interesting to find that the steady-state level of a number of transcripts involved in ethylene signaling was elevated in MBF1c-expressing

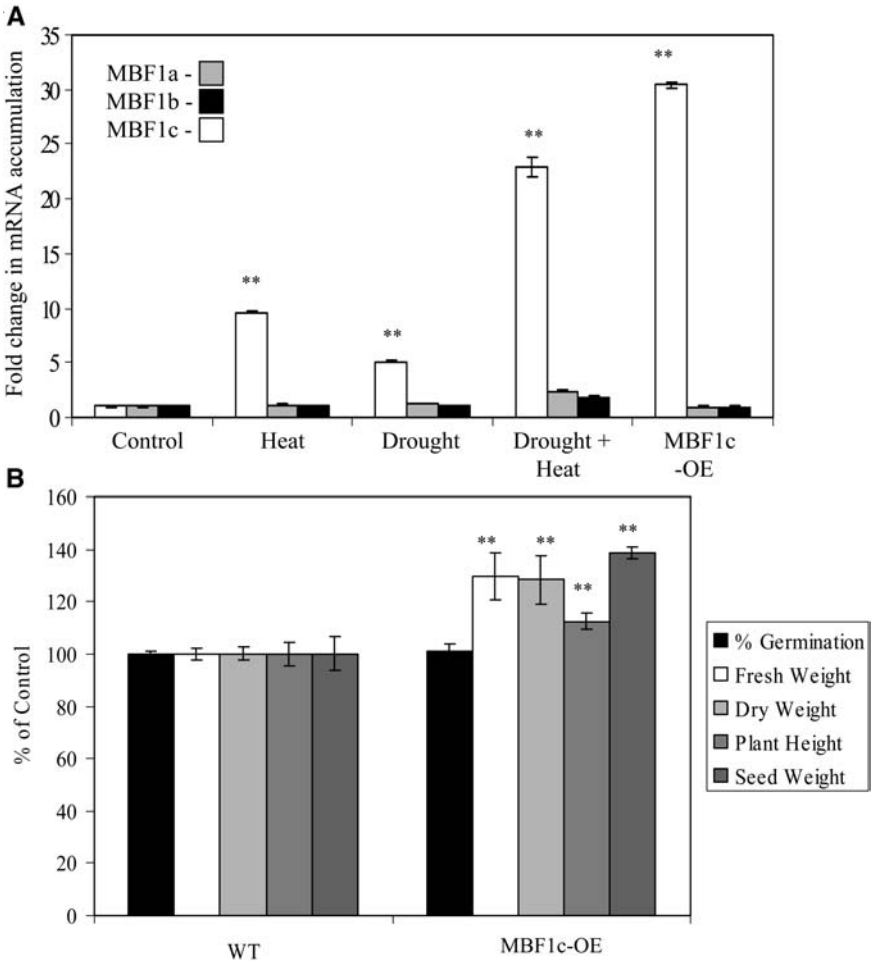

Figure 1. Characterization of transgenic plants expressing MBF1c (MBF1c-OE). A, Relative expression of MBF1 transcripts (MBF1a, At2g42680; MBF1b, At3g58680; MBF1c, At3g24500) in transgenic plants expressing MBF1c (MBF1c-OE) or wild-type plants subjected to heat stress, drought, or a combination of heat stress and drought (after Rizhsky et al. [2004b]). B, Growth and productivity of wild-type and MBF1c-expressing (MBF1c-OE) transgenic plants. Plants were grown at 21°C, 14-h light cycle, 100 μmol m^{-2} s^{-1}, and a relative humidity of 70%. Production of transgenic plants and RNA blots were performed as described in "Materials and Methods." **, Student's *t* test significant at $P < 0.01$.

plants. These include transcripts encoding ethylene-response-binding factors and the rate-limiting ethylene biosynthesis enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. Although a relatively large number of transcripts enhanced by ethylene treatment of wild-type plants (e.g. De Paepe et al., 2004) did not accumulate in transgenic plants expressing MBF1c, compared to the transcriptome of wild-type plants subjected to different abiotic stresses, ethylene-response transcripts were overrepresented in the transcriptome

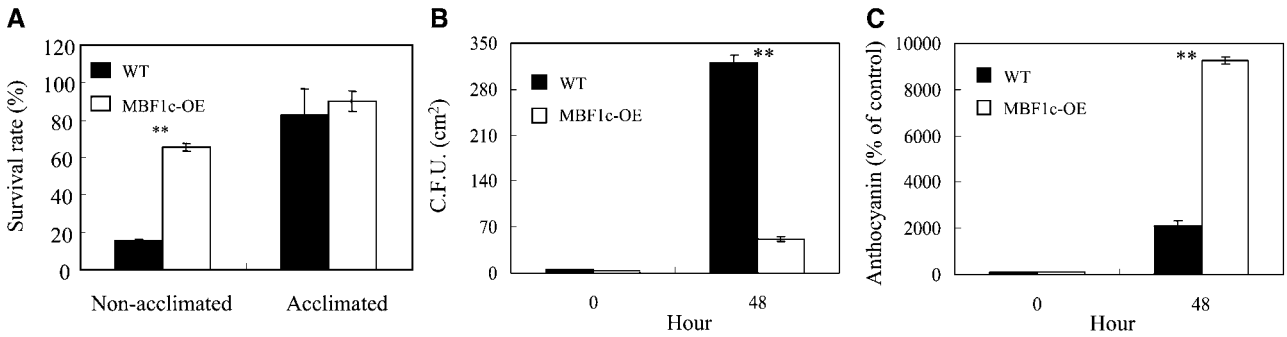

Figure 2. Enhanced tolerance of transgenic plants expressing MBF1c (MBF1c-OE) to heat stress and bacterial growth. A, Survival rates of wild-type and transgenic seedlings in response to heat stress (45°C for 2 h), showing enhanced basal thermotolerance of MBF1c-expressing plants. B, In planta bacterial population measurements showing enhanced resistance of MBF1c-expressing plants to *Pseudomonas syringae* inoculation. Bacteria (50 cfu cm^{-2} prepared in water) was infiltrated into leaves with a syringe. Forty-eight hours after inoculation, bacteria was extracted from leaves, plated on agar plates, and scored for cfu cm^{-2}. C, Augmented accumulation of anthocyanins in MBF1c-expressing plants in response to light stress (1,000 μmol m^{-2} s^{-1} for 48 h). Stress assays and pathogen infection were performed as described in "Materials and Methods." **, Student's *t* test significant at $P < 0.01$.

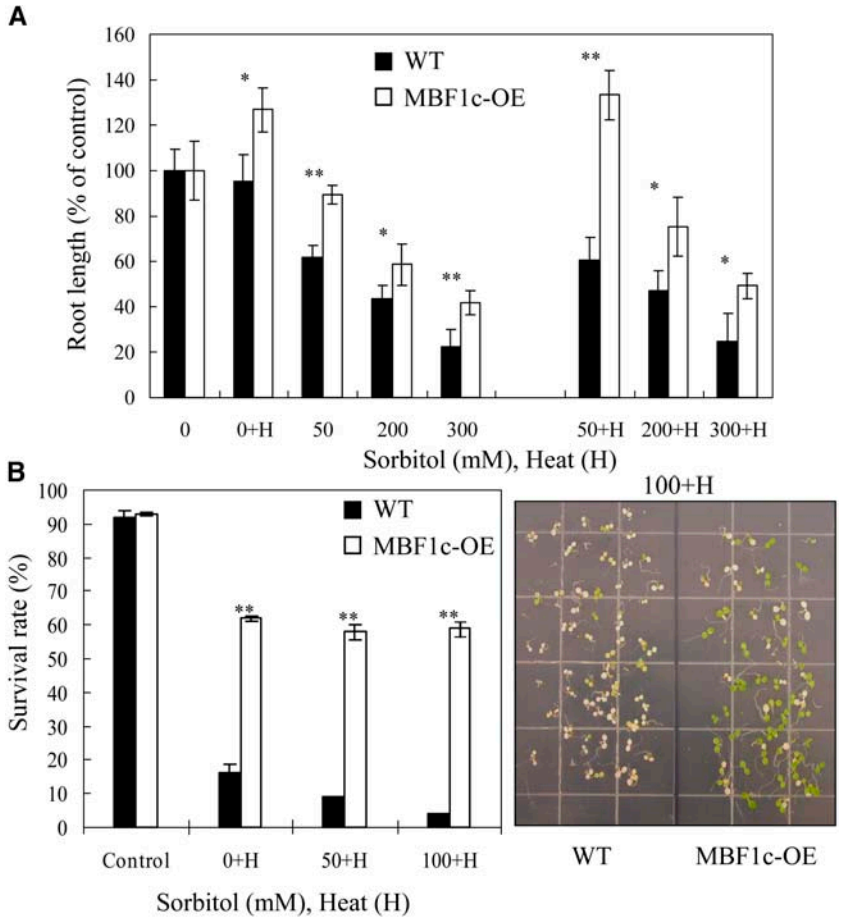

Figure 3. Enhanced tolerance of transgenic seedlings expressing MBF1c (MBF1c-OE) to heat stress, osmotic stress, or a combination of osmotic and heat stress. A, Root growth of wild-type and transgenic seedlings subjected to heat stress (38°C, 48 h), osmotic stress (sorbitol, 50, 200, and 300 mM), or their combination. B, Survival rate measurements of MBF1c-expressing seedlings subjected to heat stress (45°C for 2 h) or heat stress combined with osmotic stress (sorbitol, 50, 100 mM). C, A photograph of wild-type and transgenic seedlings subjected to heat stress (45°C for 2 h) combined with osmotic stress (sorbitol, 100 mM). Stress assays were performed as described in "Materials and Methods." **, Student's *t* test significant at $P < 0.01$; *, Student's *t* test significant at $P < 0.05$.

of transgenic plants expressing MBF1c (Table I; Supplemental Fig. 2).

MBF1c expression resulted in the accumulation of transcripts encoding pathogenesis-related (PR) proteins, such as chitinase (PR-3) and glucanase (PR-2). Expression of PR-2 and PR-3 was associated with enhanced tolerance of plants to pathogens (Mittler et al., 1995), and might explain the enhanced tolerance of MBF1c plants to bacterial growth (Fig. 2B). With the exception of transcripts encoding a protein with a DNAJ domain, transcripts encoding classical heat shock proteins (HSPs) or drought-response late embryogenesis abundant proteins did not accumulate in transgenic plants under control conditions, suggesting that the enhanced tolerance of these plants to osmotic and heat stress (Figs. 2 and 3) is not associated with constitutive expression of HSPs and late embryogenesis abundant proteins. Of the transcripts elevated in transgenic plants expressing MBF1c, only two transcripts (At3g48520-cytochrome P450 and At1g62510-lipid transfer protein) were also elevated by heat stress or a combination of heat stress and drought in wild-type plants (Table I; Rizhsky et al., 2004b). A complete list of all transcripts significantly elevated or suppressed in MBF1c-expressing plants grown under controlled conditions is included in Supplemental Tables I and II, respectively. It should be noted that the results presented in Table I were obtained with young plants grown in soil and could not be directly correlated with tolerance of 4- to 5-d-old seedlings grown on agar plates (Figs. 2 and 3).

The results presented in Table I suggest that MBF1c does not act as a classical drought- or heat-response transcription factor transgene that constitutively enhances the expression of defense transcripts involved in the response of plants to drought or heat (see e.g. Kasuga et al., 1999; Mishra et al., 2002). The effects of MBF1c on plant tolerance to environmental stress (Figs. 2 and 3) might therefore be linked to its putative coactivator function in cells (Tsuda et al., 2004). Thus, MBF1c might augment the plant's response during stress by binding to different stress-response promoters and facilitating their activation.

Augmented Response of MBF1c-Expressing Plants to Heat Stress

To test whether MBF1c expression in transgenic plants facilitates the accumulation of different stress-response transcripts during heat stress, possibly by acting as a transcriptional coactivator, we compared the accumulation of different stress-response transcripts

Table I. *Transcripts elevated in MBF1c-expressing plants under controlled conditions*

Transcripts with a putative or known function significantly elevated in transgenic plants expressing MBF1c compared to wild-type plants (cutoff >1.5 $\log_2$). ATH1 and AGI locus identification numbers are given on left. Transcript annotation and fold change in $\log_2$ are given on right. Plant growth under controlled conditions, transcriptome profiling, and data analysis were performed as described in "Materials and Methods." A complete list of all transcripts significantly elevated or suppressed in MBF1c-expressing plants (cutoff >1.5 $\log_2$) is included in Supplemental Tables I and II, respectively. Transcripts indicated by a "*" were also found to be elevated in wild-type plants in response to heat stress or a combination of drought and heat stress (Rizhsky et al., 2004b).

Array Element	Locus Identifier	Fold ($\log_2$)	SD	Annotation
Transgene				
258133_at	AT3G24500	4.70	0.40	MBF1c*
Transcription factors and signal transduction transcripts				
248400_at	AT5G52020	3.50	1.35	APETALA2 (AP2) domain transcription factor
258947_at	AT3G01830	3.07	0.12	Calmodulin-like
249197_at	AT5G42380	2.93	0.06	Similar to calmodulin
255937_at	AT1G12610	2.90	0.10	Similar to transcriptional activator CBF1
261984_at	AT1G33760	2.87	0.15	AP2 domain-containing transcription factor
262360_at	AT1G73080	2.60	1.14	Leu-rich repeat transmembrane protein kinase
259879_at	AT1G76650	2.60	0.26	Calmodulin
258682_at	AT3G08720	2.47	0.45	Ser/Thr protein kinase (PK19)
264147_at	AT1G02205	2.20	0.17	Receptor-like protein glossy1 (gl1)
255511_at	AT4G02075	2.17	0.31	Zinc finger (C3HC4-type RING finger)
257919_at	AT3G23250	2.17	0.16	Myb family transcription factor (MYB15)
249862_at	AT5G22920	2.13	0.12	Zinc finger (C3HC4-type RING finger)
261892_at	AT1G80840	1.97	0.21	WRKY40
251745_at	AT3G55980	1.93	0.12	Zinc-finger transcription factor (PEI1)
247137_at	AT5G66210	1.90	0.17	Calcium-dependent protein kinase
267028_at	AT2G38470	1.87	0.15	WRKY33
253915_at	AT4G27280	1.87	0.06	Calcium-binding EF hand protein
253485_at	AT4G31800	1.87	0.15	WRKY18
248389_at	AT5G51990	1.83	0.31	DRE-binding protein/CRT/DRE-binding factor
252193_at	AT3G50060	1.80	0.10	R2R3-MYB transcription factor
250099_at	AT5G17300	1.77	0.29	Myb family transcription factor
257022_at	AT3G19580	1.77	0.12	Zinc-finger (C2H2 type) protein 2
246028_at	AT5G21170	1.77	0.06	5′-AMP-activated protein kinase
265737_at	AT2G01180	1.73	0.21	Phosphatidic acid phosphatase family protein
259428_at	AT1G01560	1.70	0.26	Mitogen-activated protein kinase 11
260856_at	AT1G21910	1.70	0.26	AP2 domain-containing transcription factor
245247_at	AT4G17230	1.70	0.20	Scarecrow-like transcription factor 13
251259_at	AT3G62260	1.70	0.17	Protein phosphatase 2C
258188_at	AT3G17790	1.70	0.10	Acid phosphatase type 5
255568_at	AT4G01250	1.67	0.15	WRKY family transcription factor
267460_at	AT2G33810	1.67	0.12	Squamosa-promoter binding protein like 3
248606_at	AT5G49450	1.60	0.10	bZIP transcription factor
246777_at	AT5G27420	1.60	0.10	RING-H2 zinc finger protein
267083_at	AT2G41100	1.57	0.12	Touch-responsive, calmodulin-related protein 3
263783_at	AT2G46400	1.57	0.06	WRKY46
247426_at	AT5G62570	1.53	0.15	Calmodulin-binding protein
252592_at	AT3G45640	1.50	0.17	Mitogen-activated protein kinase 3
251636_at	AT3G57530	1.50	0.10	Calcium-dependent protein kinase
Ethylene-associated transcripts				
253259_at	AT4G34410	2.93	0.15	Ethylene-responsive element binding
257918_at	AT3G23230	2.70	0.12	Ethylene-responsive element binding protein 4
266821_at	AT2G44840	2.13	0.15	Ethylene-response element binding protein
245250_at	AT4G17490	2.07	0.12	Ethylene-responsive element binding factor-like protein 6
261470_at	AT1G28370	1.90	0.10	Ethylene-responsive element binding factor
248448_at	AT5G51190	1.83	0.12	Ethylene-responsive element binding factor
254926_at	AT4G11280	1.70	0.06	ACC synthase 6
248799_at	AT5G47230	1.50	0.17	Ethylene-responsive element binding factor 5
Pathogen and stress-associated transcripts				
253161_at	AT4G35770	3.60	0.35	Senescence-associated, sen1
245757_at	AT1G35140	3.13	0.15	Phosphate-responsive protein phi-1
252368_at	AT3G48520	2.70	0.10	Cytochrome P450*

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Array Element	Locus Identifier	Fold (log_2)	SD	Annotation
264153_at	AT1G65390	2.40	0.20	Disease resistance protein RPS4
261037_at	AT1G17420	2.30	0.10	Lipoxygenase
260399_at	AT1G72520	2.27	0.21	Lipoxygenase
255064_at	AT4G08950	2.27	0.06	Phosphate-responsive protein
266072_at	AT2G18700	2.23	0.06	Trehalose-6-P synthase
248964_at	AT5G45340	2.20	0.10	Cytochrome P450
256763_at	AT3G16860	2.17	0.15	Phytochelatin synthetase related
249645_at	AT5G36910	2.13	0.15	Thionin Thi2.2
246099_at	AT5G20230	2.07	0.12	Blue copper-binding protein
251804_at	AT3G55430	2.00	0.10	β-1,3-Glucanase PR-2
246114_at	AT5G20250	1.93	0.06	Raffinose synthase
264213_at	AT1G65390	1.90	0.26	Disease resistance protein (TIR class)
265648_at	AT2G27500	1.87	0.15	β-1,3-Glucanase PR-2
263019_at	AT1G23870	1.83	0.72	Glycosyl transferase/trehalose-phosphatase
247280_at	AT5G64260	1.83	0.12	Phosphate-responsive protein phi-1
265111_at	AT1G62510	1.80	0.10	Protease inhibitor/seed storage/lipid transfer*
261901_at	AT1G80920	1.80	0.00	DNAJ N-terminal domain protein
256526_at	AT1G66090	1.77	0.06	Disease resistance protein, RPP1-WsA
247279_at	AT5G64310	1.70	0.10	Arabinogalactan protein 1
261443_at	AT1G28480	1.67	0.12	Glutaredoxin
262382_at	AT1G72920	1.63	0.15	Disease resistance protein (TIR-NBS class)
249746_at	AT5G24590	1.60	0.26	Turnip crinkle virus-interacting protein
245668_at	AT1G28330	1.60	0.10	Dormancy-associated protein, putative
259443_at	AT1G02360	1.57	0.25	Chitinase PR-3
249264_s_at	AT5G41740	1.57	0.21	Disease resistance protein (TIR-NBS-LRR class)
264339_at	AT1G70290	1.57	0.15	Trehalose-6-P synthase
Metabolism, development, and cell structure				
248622_at	AT5G49360	3.60	0.10	Glycosyl hydrolase family 3 protein
247866_at	AT5G57550	2.23	0.15	Xyloglucan:xyloglucosyl transferase
262456_at	AT1G11260	2.13	0.06	Glucose transporter (STP1)
256300_at	AT1G69490	2.10	0.26	No apical meristem family protein
256772_at	AT3G13750	2.07	0.31	β-Galactosidase, putative/lactase
265680_at	AT2G32150	1.83	0.21	Haloacid dehalogenase-like hydrolase
251774_at	AT3G55830	1.80	0.10	Glycosyltransferase family protein 47
259445_at	AT1G02400	1.80	0.30	Gibberellin 2-oxidase, putative
265511_at	AT2G05540	1.77	0.06	Gly-rich protein
249742_at	AT5G24490	1.67	0.06	30S ribosomal protein
260914_at	AT1G02640	1.67	0.21	Glycosyl hydrolase family 3 protein
263443_at	AT2G28630	1.63	0.06	β-Ketoacyl-CoA synthase family protein

during heat stress between wild-type and transgenic plants. For this purpose we specifically chose heat- or oxidative-stress-response transcripts that were not elevated in MBF1c-expressing plants in the absence of stress (i.e. absent from Table I). The choice of oxidative-stress-response transcripts was based on a recent report in which MBF1 was shown to regulate the redox response of AP-1 during oxidative stress in Drosophila (Jindra et al., 2004). As shown in Figure 4, in response to heat stress, transcripts encoding the defense proteins ascorbate peroxidase 2 (APX2), ferritin, and the zinc-finger proteins Zat7 and Zat12 accumulated in MBF1c-expressing plants to a higher level than in wild-type plants. In contrast, transcripts encoding APX1 and different HSPs accumulated to a similar level in wild-type and transgenic plants. Constitutive expression of ferritin, Zat12, or Zat7 was shown to enhance the tolerance of transgenic plants to biotic and abiotic stresses (Deak et al., 1999; Rizhsky et al., 2004a). It is possible that the augmented accumulation of these transcripts during stress in transgenic plants (Fig. 4) contributes to the enhanced tolerance of transgenic plants to abiotic stresses (Figs. 2 and 3).

Sugars such as Suc or trehalose play a key role in plant tolerance to drought and heat stress and might have a protective or stabilizing role that could enhance stress tolerance (Bray et al., 2000; Cushman and Bohnert, 2000; Garg et al., 2002; Kaplan et al., 2004; Rizhsky et al., 2004b). Comparative profiling of sugars in transgenic and wild-type plants subjected to heat stress (Supplemental Fig. 3) revealed that the relative level of trehalose was higher in MBF1c-expressing plants compared to wild-type plants under controlled conditions. The relative level of trehalose was further enhanced in transgenic plants in response to heat stress (Supplemental Fig. 3). The higher level of treahalose and its enhanced accumulation during heat stress in transgenic plants could be linked to the accumulation of transcripts encoding trehalose-6-P synthase in transgenic MBF1c plants grown under

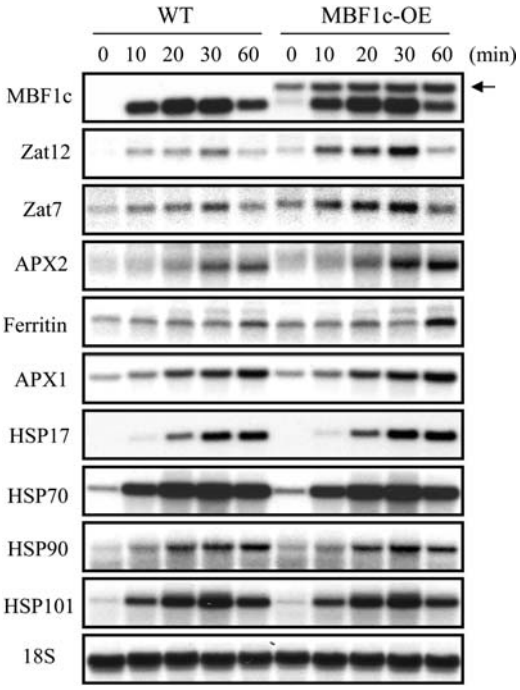

Figure 4. Augmented response of MBF1c-expressing plants (MBF1c-OE) to heat stress. A time-course RNA gel-blot analysis of 2-week-old wild-type and MBF1c-expressing plants subjected to heat stress (38°C, 10, 20, 30, and 60 min), showing the augmented accumulation of transcripts encoding Zat12, Zat7, APX2, and ferritin in transgenic plants. Time-course experiments were repeated three times with similar results. Representative RNA blots are shown. RNA blots and stress assays were performed as described in ''Materials and Methods.'' Plants were grown at 21°C, 14-h light cycle, 100 μmol m^{-2} s^{-1}, and a relative humidity of 70% and subjected to heat stress as described above. Arrow on right side of top section indicates the transgenic transcript of MBF1c. **, Student's *t* test significant at $P < 0.01$.

controlled conditions (i.e. At2g18700 and At1g70290; Table I). Trehalose overaccumulation was shown to enhance the tolerance of transgenic plants to abiotic stresses (Garg et al., 2002; Penna, 2003). These findings might explain the enhanced tolerance of MBF1c plants to abiotic stresses (Figs. 2 and 3). Further studies, including direct measurements of trehalose biosynthesis and degradation in transgenic plants, are, however, required to elucidate the role of trehalose in enhancing the tolerance of MBF1c-expressing plants to abiotic stresses.

MBF1c Expression Enhances the Tolerance of Transgenic Plants to Abiotic Stresses by Partially Activating, or Perturbing, the Ethylene-Response Signal Transduction Pathway

The accumulation of transcripts associated with ethylene signaling in transgenic plants (Table I; Supplemental Fig. 2) suggests that MBF1c expression partially activates, or perturbs, the ethylene-response signal transduction pathway. To test this possibility, we examined whether etiolated seedlings of transgenic plants exhibit the classical triple response associated with ethylene perception (Guzman and Ecker, 1990). As shown in Figure 5A, etiolated seedlings of MBF1c-expressing plants, when compared to wild type, exhibited a stronger triple-response phenotype in the presence or absence of ACC. This result could suggest that MBF1c expression enhances the biosynthesis of ethylene in transgenic plants. Alternatively, MBF1c expression could enhance the sensitivity of transgenic plants to ethylene. To test whether ethylene signaling is involved in the enhanced tolerance of MBF1c-expressing plants to osmotic or heat stress, we tested the effects of the ethylene-signaling inhibitor aminoethoxyvinylglycine (AVG) or silver thiosulfate (STS) on wild-type and transgenic plants subjected to heat stress or heat stress combined with osmotic stress. As shown in Figure 5B, AVG application suppressed the tolerance of MBF1c-expressing plants to heat stress or heat stress combined with osmotic stress. Similar results were obtained with STS (data not shown). To test whether ethylene signaling is required for plant tolerance to a heat stress, osmotic stress, or a combination of heat and osmotic stress, we compared wild-type plants to *ein-2* mutants, impaired in ethylene sensing (Guzman and Ecker, 1990). As shown in Figure 5C, *ein-2* mutants are more sensitive than wild type to these stresses.

DISCUSSION

Enhancing plant tolerance to biotic or abiotic stress conditions by activating a stress-response signal transduction pathway in transgenic plants is a powerful and promising approach (Kasuga et al., 1999; Cushman and Bohnert, 2000; Kovtun et al., 2000; Umezawa et al., 2004). Here we report that constitutive expression of the eukaryotic transcriptional coactivator MBF1c in Arabidopsis enhances the tolerance of transgenic plants to bacterial infection, salinity, heat, and osmotic stress, and that the enhanced tolerance of transgenic plants to heat or osmotic stress is maintained even when these two stresses are combined. We further show that MBF1c expression enhances the tolerance of transgenic plants to heat and osmotic stress by perturbing, or partially activating, the ethylene-response signal transduction pathway. Evidence supporting this finding include the accumulation of different ethylene-response transcripts as well as transcripts encoding ACC synthase in MBF1c-expressing plants (Table I), the partial triple-response phenotype of etiolated MBF1c-expressing seedlings (Fig. 5A), and the inhibition of MBF1c-induced tolerance to stress by inhibitors of the ethylene response (Fig. 5B). In contrast to examples in which enhanced tolerance to abiotic stresses was associated with suppressed growth of transgenic plants (Kasuga et al., 1999), constitutive expression of MBF1c did not suppress plant growth (Fig. 1B). The accumulation of MBF1c-encoding transcripts in different plants in response to drought, heat stress, and a combination of drought and heat stress (Fig. 1A;

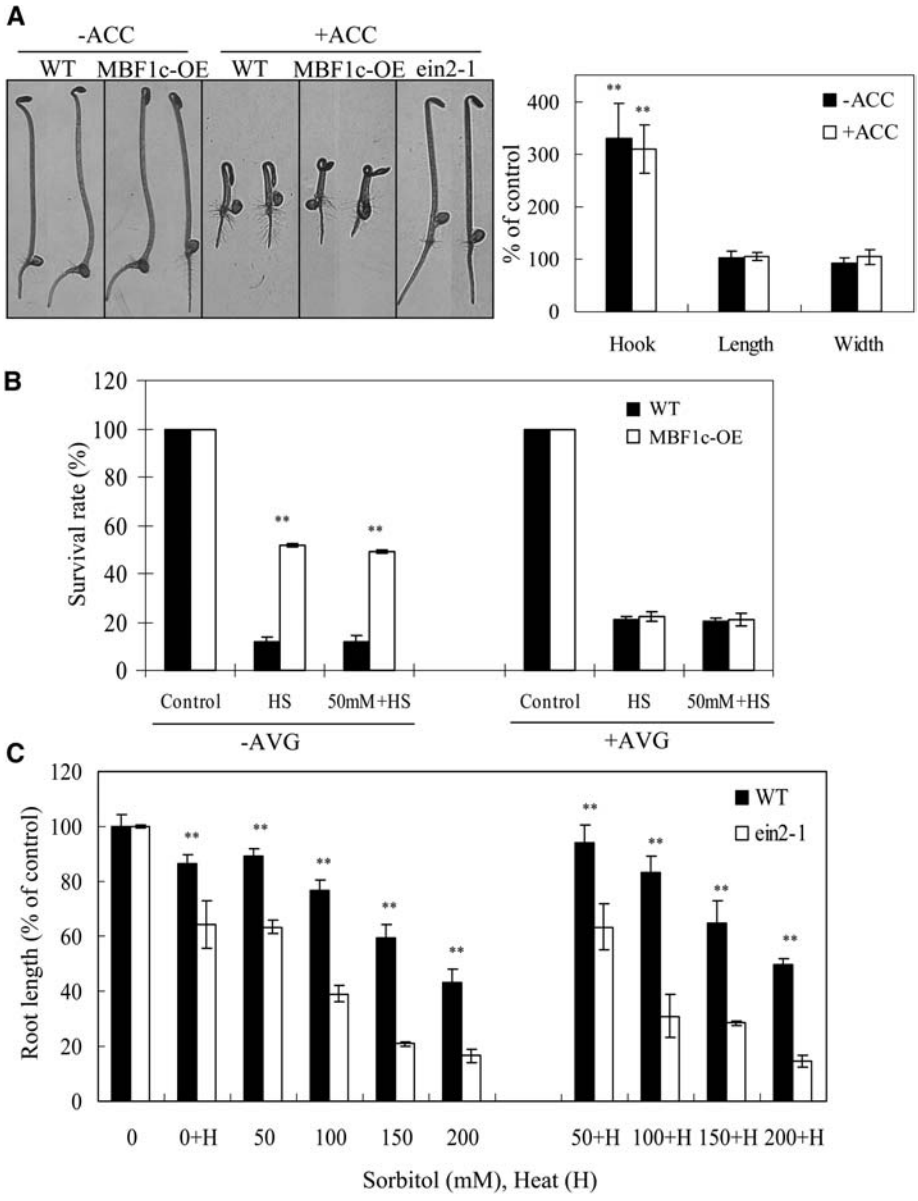

Figure 5. Enhanced tolerance to abiotic stress in MBF1c-expressing plants is mediated by ethylene signaling. A, Triple-response phenotype of etiolated MBF1c-expressing seedlings in the presence or absence of ACC compared to wild type. B, Survival rate measurements showing suppression of MBF1c-induced tolerance to abiotic stress by the ethylene-signaling inhibitor AVG. C, Root growth measurements of *ein2* seedlings, impaired in ethylene sensing, showing enhanced sensitivity to osmotic and heat stress compared to wild type. Stress assays, application of AVG, and *ein2* analysis were performed as described in "Materials and Methods." **, Student's *t* test significant at $P < 0.01$.

Pnueli et al., 2002; Rizhsky et al., 2002, 2004b); the augmented response of transgenic plants to heat stress (Fig. 4); and the enhanced tolerance of transgenic plants to osmotic stress and heat stress (Figs. 2 and 3) suggest that MBF1 proteins could potentially be used to enhance the tolerance of different plants and crops to these stresses. The interpretation of the results obtained with osmotic and heat stress combination on agar plates (Figs. 2 and 3) should, however, take into consideration the high humidity conditions that occur during these assays. These do not reflect the conditions that occur in the field and may decrease the effects of the osmotic stress on plant acclimation to the stress combination.

Expression of MBF1c in transgenic plants resulted in the constitutive expression of several signal transduction and defense transcripts (Table I), as well as in the augmented accumulation of different stress-associated transcripts in response to heat (Fig. 4). These findings suggest that constitutive expression of MBF1c in transgenic plants alters the accumulation of specific transcripts under controlled conditions and during stress. It is possible that in transgenic plants, MBF1c links between different transcription factors and the basal transcriptional machinery to form a complex that associates with, and activates, specific promoters. The transcripts identified by our study as hyperresponsive or constitutively expressed in transgenic plants (Fig. 4; Table I), might be ideal subjects for future studies to address this possibility. The finding that only a small part of the defense response of plants against drought, heat stress, or a combination of drought and heat stress (Rizhsky et al., 2004b) is constitutively activated in MBF1c-expressing plants grown under controlled

conditions (Table I) suggests that augmentation of defense responses by MBF1c during stress (Fig. 4), rather than constitutive activation of defenses (Table I), is the main mode of action of MBF1c in transgenic plants. Compared to the broad effects on gene expression and plant development reported in transgenic plants expressing a transcriptional coactivator that affects histone acetylation (Stockinger et al., 2001; Vlachonasios et al., 2003), the effects of MBF1c expression in transgenic plants appeared to be more limited (Fig. 1; Table I), suggesting that MBF1c binds to specific promoters mainly associated with stress or pathogen responses.

Developing plants with enhanced tolerance to different abiotic stresses and their combination is essential for agricultural production worldwide (Boyer, 1982; Cushman and Bohnert, 2000; Moffat, 2002). Our analyses of transgenic plants expressing MBF1c demonstrate that this transcriptional coactivator plays an important role in plant protection against different environmental stresses. In addition, at least with osmotic and heat stress, the tolerance MBF1c induces in plants toward these stresses was maintained even when they were combined. Our findings thus offer a transgenic strategy to develop plants and crops with enhanced tolerance to different abiotic stresses.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Molecular Analysis

Arabidopsis plants (*Arabidopsis thaliana* cv Columbia) were grown in peat pellets (Jiffy-7, Shippagan) under controlled conditions: 21°C, 14-h light cycle, 100 μmol m^{-2} s^{-1}, and a relative humidity of 70% (E-30, AR-66; Percival Scientific). Plant transformation was performed with the binary vector pB001 as described by Rizhsky et al. (2004a). Transgenic plants were selected based on herbicide tolerance (*bar*) and screened by RNA blots. T_4 homozygous lines pooled from three independent transformation events were used for this study (Supplemental Fig. 1). RNA was isolated and analyzed as described previously (Davletova et al., 2005). Sugars were isolated and analyzed by gas chromatography-mass spectrometry as described by Rizhsky et al. (2004b). For the analysis of transcript accumulation in response to heat stress, 2-week-old plants were heat stressed at 38°C, 100 μmol m^{-2} s^{-1}, and sampled at 0, 10, 20, 30, and 60 min. All experiments were performed in triplicates and repeated at least three times.

DNA Chip Analysis

In three independent experiments, RNA was isolated from 17-d-old control and MBF1c-expressing plants grown under controlled conditions as described above. All experiments were sampled at the same time of day (10 AM). At least 75 plants were used for each RNA sample, and RNA was isolated using Trizol. RNA samples were used to perform chip hybridization analyses (Arabidopsis ATH1 chips; Affymetrix) at the University of Iowa DNA facility (http://dna9.intmed.uiowa.edu/microarrays.htm). Conditions for RNA isolation, labeling, and hybridization are described by Davletova et al. (2005). All GeneChip arrays were processed first by robust multi-array average (RMA; Irizarry et al., 2003) using the *R* package affy (Gautier et al., 2004). Specifically, expression values were computed from raw *CEL* files by first applying the RMA model of probe-specific correction of perfect-match probes. These corrected probe values were then normalized via quantile normalization, and a median polish was applied to compute one expression measure from all probe values. Resulting RMA expression values were $\log_2$ transformed. These are standard methods for processing Affymetrix data. Please see the affy manual at www.bioconductor.org/repository/devel/vignette/affy.pdf for details. Density plots and boxplots of RMA expression value distributions of all arrays were very similar with no apparent outlying arrays (data not shown). Digestion curves describing trends in RNA degradation between the 5′ end and the 3′ end in each probeset were generated, and all six proved very similar, with a downward trend at the 5′ end (data not shown). To determine whether genes were differentially expressed, an ANOVA was performed on the RMA expression values. For an overview on the application of ANOVA to microarray data, please see Kerr et al. (2000). The model described in Davletova et al. (2005) was used for this analysis, and transcripts with adjusted P values < 0.05 were extracted for further analysis. Of these, genes with differential expression of more than 1.5 log2 were selected. The *R* package limma was used for ANOVA methods (www.bioconductor.org/repository/devel/vignette/affy.pdf). The experiments described in this paper were performed side-by-side with the experiments reported by Rizhsky et al. (2004b) and could therefore be compared to these experiments. Microarray data from this experiment were submitted to the Nottingham Arabidopsis Stock Centre arrays at http://affymetrix.arabidopsis.info/.

Stress Assays and Application of Ethylene Inhibitors

Bacterial (*Pseudomonas syringae* cv tomato) infection, light stress, and anthocyanin measurements were performed as described previously (Mittler et al., 1997, 1999; Bariola et al., 1999; Pnueli et al., 2003). To avoid complications resulting from differences in plant size and reproduction stage, all stress assays were performed with 4- to 5-d-old wild-type and transgenic seedlings, or 14-d-old wild-type and transgenic plants. At these growth stages no differences were observed between the size and developmental stage of wild-type or transgenic plants (data not shown). In addition, the stress tolerance of MBF1c-expressing plants was compared to that of wild-type plants, as well as that of empty vector plants. No differences were found between the tolerance of wild-type plants and empty vector plants (data not shown). For the analysis of abiotic stress tolerance, seeds of wild-type and MBF1c-expressing lines were surface sterilized and placed in rows on 1% agar plates (0.5× Murashige and Skoog [MS] medium), containing different concentrations of NaCl (50, 100, 150, and 200 mM) or sorbitol (50, 100, 200, and 300 mM). Each row of seeds placed on a plate was divided into two parts: wild type and MBF1c expressing. Thus, the different seeds were placed side by side on the same plate. Plates were incubated for 48 h at 4°C, placed vertically in a growth chamber (21°C–22°C, constant light, 100 μmol m^{-2} s^{-1}) and scored for percentage of germination and root length 5 d following transfer to the growth chamber. Four- or 5-d-old seedlings grown on 0.5× MS agar plates in a growth chamber were also subjected to heat stress (38°C, 1, 6, 24, and 48 h) and scored for root length 1 d following recovery from heat stress. For stress combination, seeds were surface sterilized and germinated on MS plates containing sorbitol or NaCl as described above and maintained vertically in a growth chamber. Five- to 7-d-old seedlings (grown on MS plates or plates supplemented with sorbitol or NaCl) were heat stressed (38°C) for 48 h and returned to controlled growth condition. Control seedlings (grown on MS plates or MS plates supplemented with sorbitol or NaCl) were maintained under controlled growth conditions. Root length was measured for all seedlings prior to the heat stress treatment and 1 d following heat stress. At least six different plates were used for each condition with approximately 30 seedlings per plate.

To measure survival rate in seedlings subjected to osmotic stress, heat stress, and their combination, surface-sterilized seeds were germinated on MS plates containing different concentrations of sorbitol (0, 50, 100 mM). Each plate was divided into two halves, and approximately 100 seeds of wild-type or MBF1c-expressing lines were spread on each of the different halves. Plates were maintained horizontally in a growth chamber as described above. Seedlings were untreated, acclimated at 38°C for 1.5 h, and treated at 45°C for 2 h, or directly treated at 45°C for 2 h without acclimation. Following heat stress, plates were incubated at 21°C for 2 to 4 d and scored for survival rate. To examine the effects of ethylene-signaling inhibitors on the response of wild-type and transgenic plants to abiotic stresses, water, or AVG (10 μM) or STS (10 μM) prepared in water, were applied to plates by spraying 30 min prior to the heat stress treatment. At least six different plates were used for each condition with approximately 200 seedlings per plate. A Student's t test was used to determine statistical significance.

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