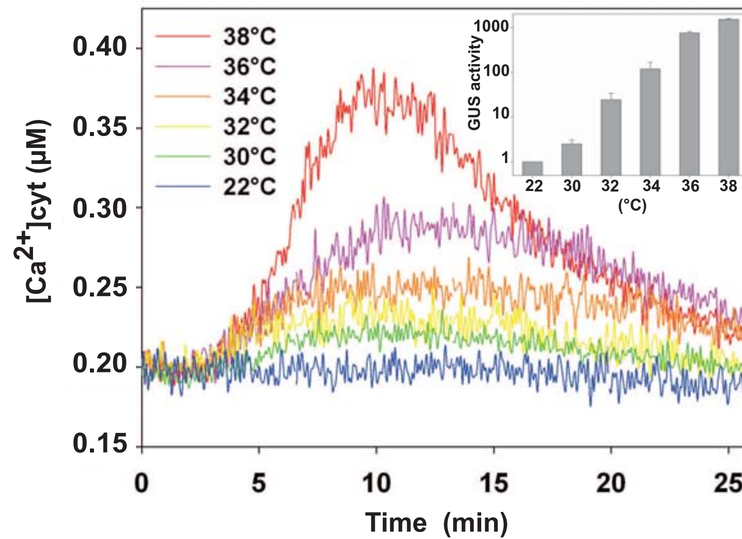
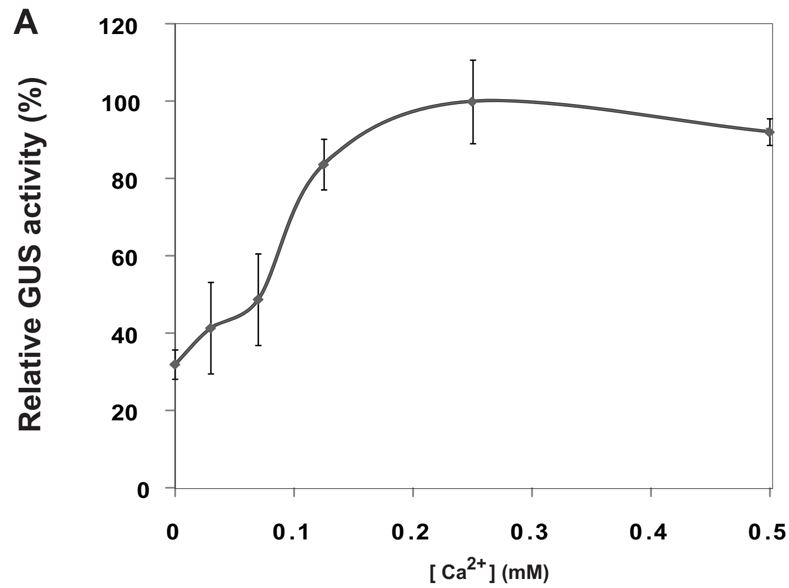
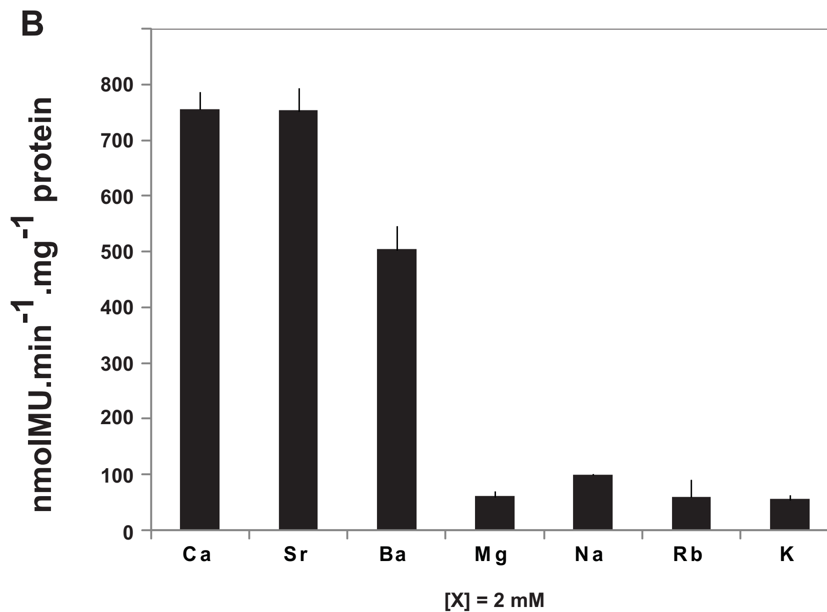
Supplemental data, Saidi et al., (2009) The Heat Shock Response in Moss Plants is Regulated by Specific Calcium-Permeable Channels in the Plasma Membrane.

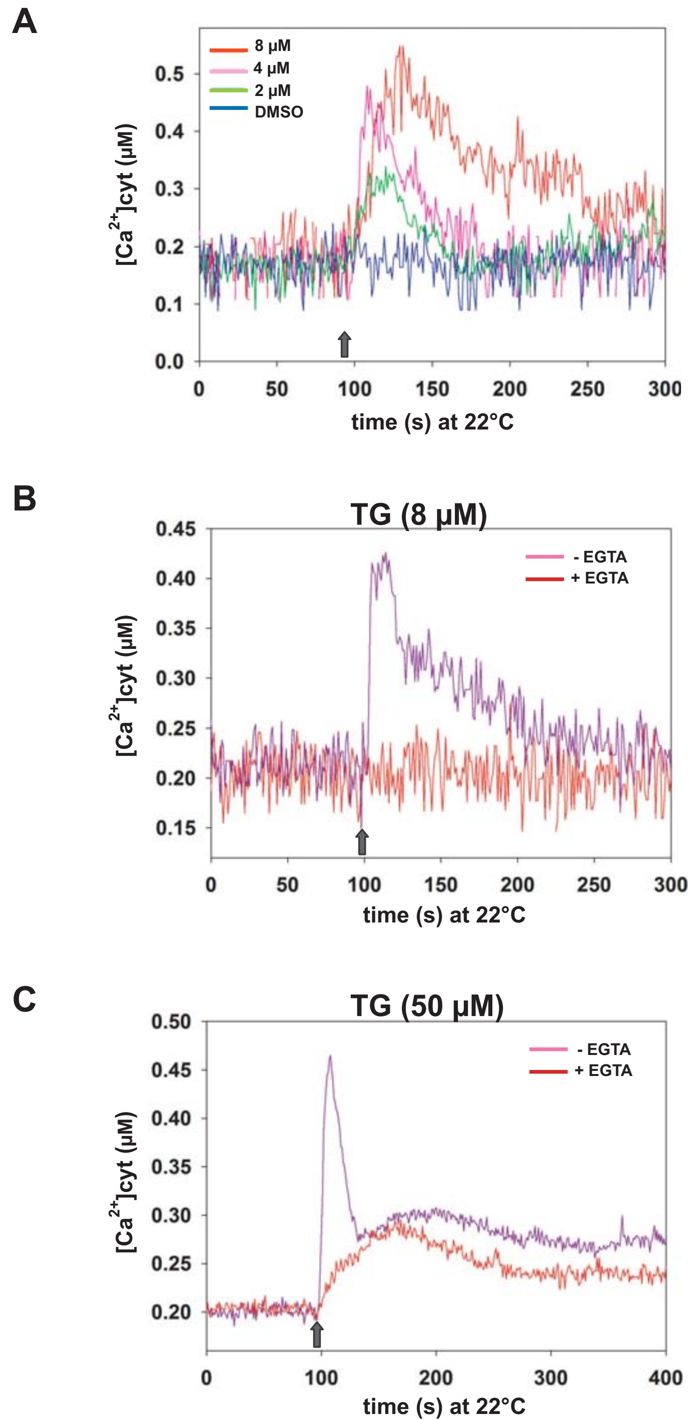


Supplemental Figure 1: The intensity of the temperature-induced Ca^{2+}-influx correlates with subsequent levels of HSR. UBI-AEQ tissues were treated for 25 min at indicated temperatures and the concentration of cytoplasmic Ca^{2+} was measured as in figure 2D. Inset, HSP-GUS tissues were treated for 60 min at indicated temperatures and the GUS activities were measured after 8h at 22°C. GUS values are means of three independent experiments and standard deviations are shown.

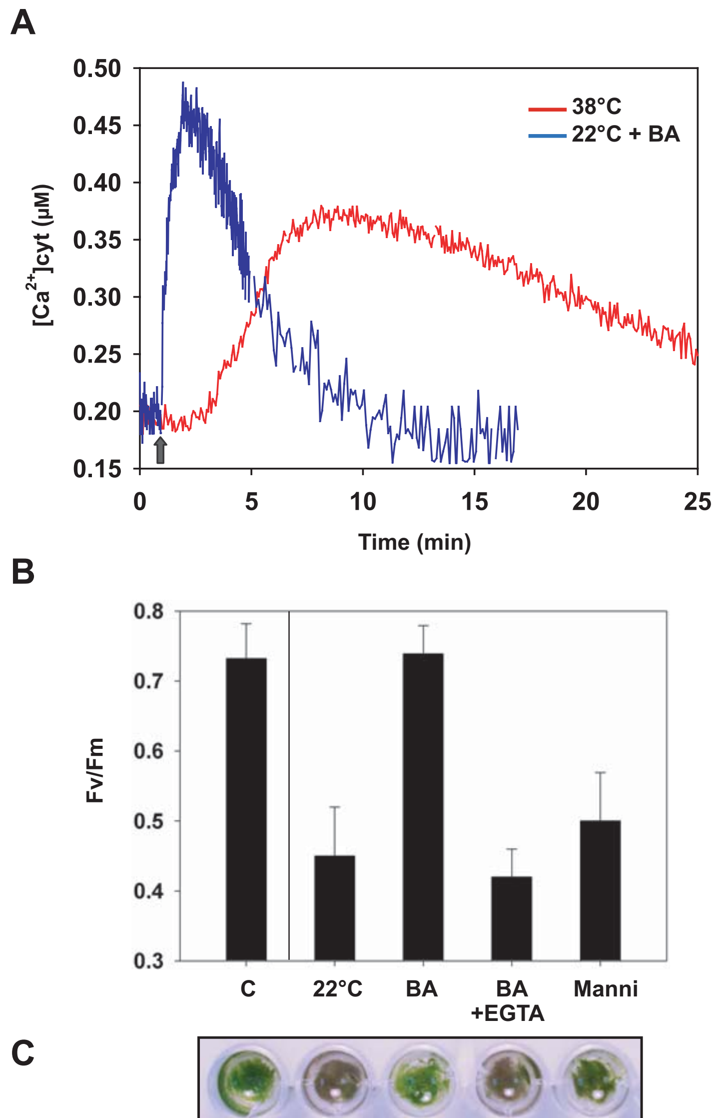




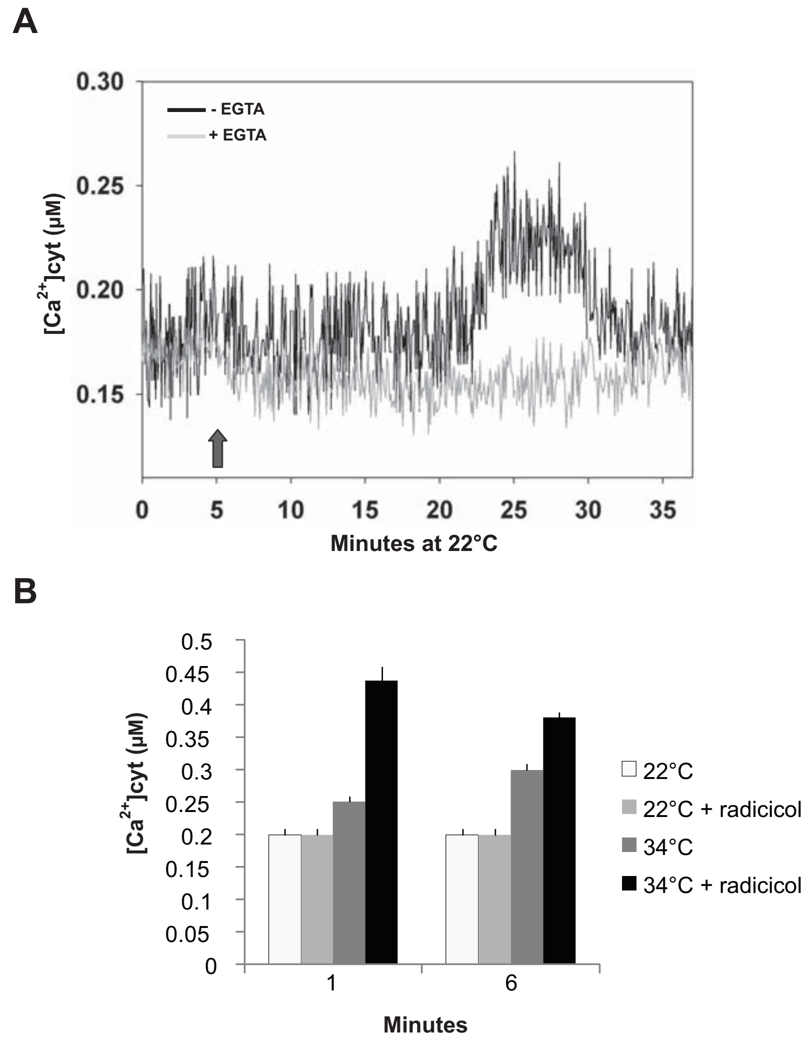
Supplemental Figure 2: Effect of increasing concentrations of Ca^{2+}, or other ions, on moss HSR. (A) HSP-GUS tissues were pre-incubated 1 hour in 7 mM EGTA at 22°C, washed 3 times in distilled water, then treated 1h at 38°C in the presence of increasing concentration of CaCl2. **(B)** HSP-GUS tissues were treated 1h at 38°C in the presence of 7 mM EGTA supplemented with 2 mM $CaCl_2$, $SrCl_2$, $BaCl_2$, $MgCl_2$, NaCl, RbCl or KCl. GUS activities were measured 8h after HS. All values are means of at least three independent experiments and standard deviations are shown.



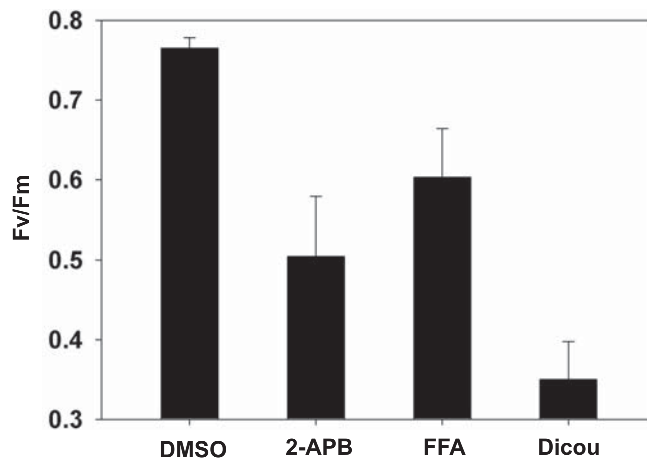
Supplemental Figure 3: Thapsigargin concentrations below 50 µM could not release Ca^{2+} from intracellular stores. (A) Effect of increasing thapsigargin (TG) concentrations on $[Ca^{2+}]_{cyt}$ at 22°C. Thapsigargin was added (arrow) to UBI-AEQ line incubated in distilled water and the cytosolic Ca^{2+} concentration was monitored every second. **(B)** Ca^{2+} transient after addition of 8 µM thapsigargin (arrow) to UBI-AEQ tissues incubated in distilled water (- EGTA) or supplemented with 7 mM EGTA (+ EGTA). **(C)** Ca^{2+} transient after addition of 50 µM thapsigargin (arrow) to UBI-AEQ tissues incubated in distilled water (- EGTA) or supplemented with 7 mM EGTA (+ EGTA).



Supplemental Figure 4: BA pre-treatment enhances thermotolerance in *P. patens*. (A) Comparison of Ca^{2+}-influxes in UBI-AEQ tissues heat-treated at 38°C (red line) or maintained at 22°C with addition (arrow) of 25 mM BA (blue line). **(B)** BA pre-treatment enhances thermotolerance. Moss tissues were first pre-treated with 25 mM BA (with or without EGTA 7mM) or 8.5% mannitol for 1 hour. After 4 hours recovery (chemicals washed out), a strong HS (1h at 42°C) was applied and F_v/F_m ratio measured. Thermotolerance was compared to non-primed tissues (22°C). "c" refers to the optimal F_v/F_m value from untreated tissues maintained at 22°C. **(C)** Following 4 hours recovery after pre-treatment as in (B), tissues were exposed to 43°C for 2 hours. Cell death was then recorded and picture taken after 3 days. The F_v/F_m values are means of three independent experiments and standard deviations are shown.

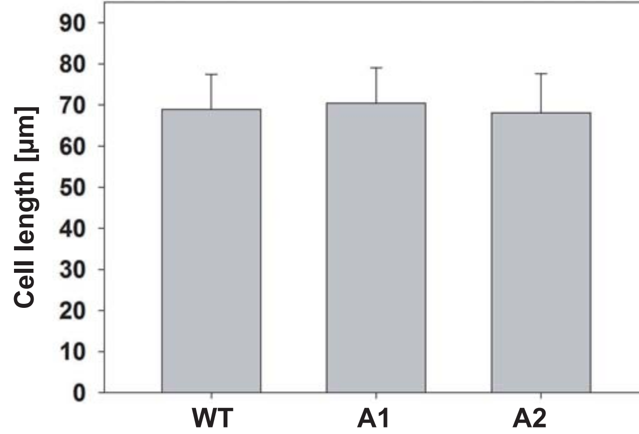


Supplemental Figure 5: Radicicol induces a transient elevation of $[Ca^{2+}]$cyt. (A) In vivo effect of radicicol on $[Ca^{2+}]$cyt. Radicicol (16 µM) was added (arrow), at the 5th min at 22°C, to UBI-AEQ tissues pre-incubated without (black line) or with (gray line) EGTA (7mM). **(B)** Comparison of relative Ca^{2+} levels in UBI-AEQ tissues exposed to 8 µM radicicol, either at 22°C, or following 1 and 6 minutes of temperature rise to 34°C. The represented values are means of three independent experiments and standard deviations are shown.



Supplemetal Figure 6: Effect of HSR inhibitors on plant acquired thermotolerance. Moss tissues were primed for 1h at 36°C in the presence of 20 μM 2-ABP, FFA, Dicoumarol or 0.3 % DMSO as in figure 7D. After four hours recovery (chemicals washed out), tissues were submitted to 1h at 42°C and F_v/F_m values were measured. Represented values are means of three independent experiments and standard deviations are shown.

A



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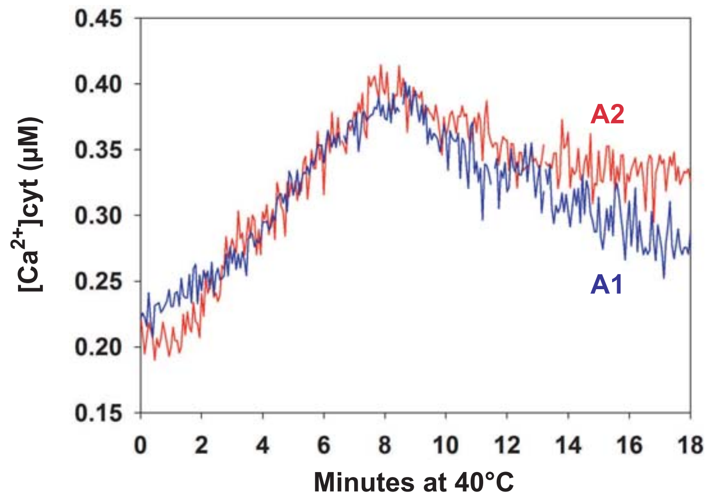
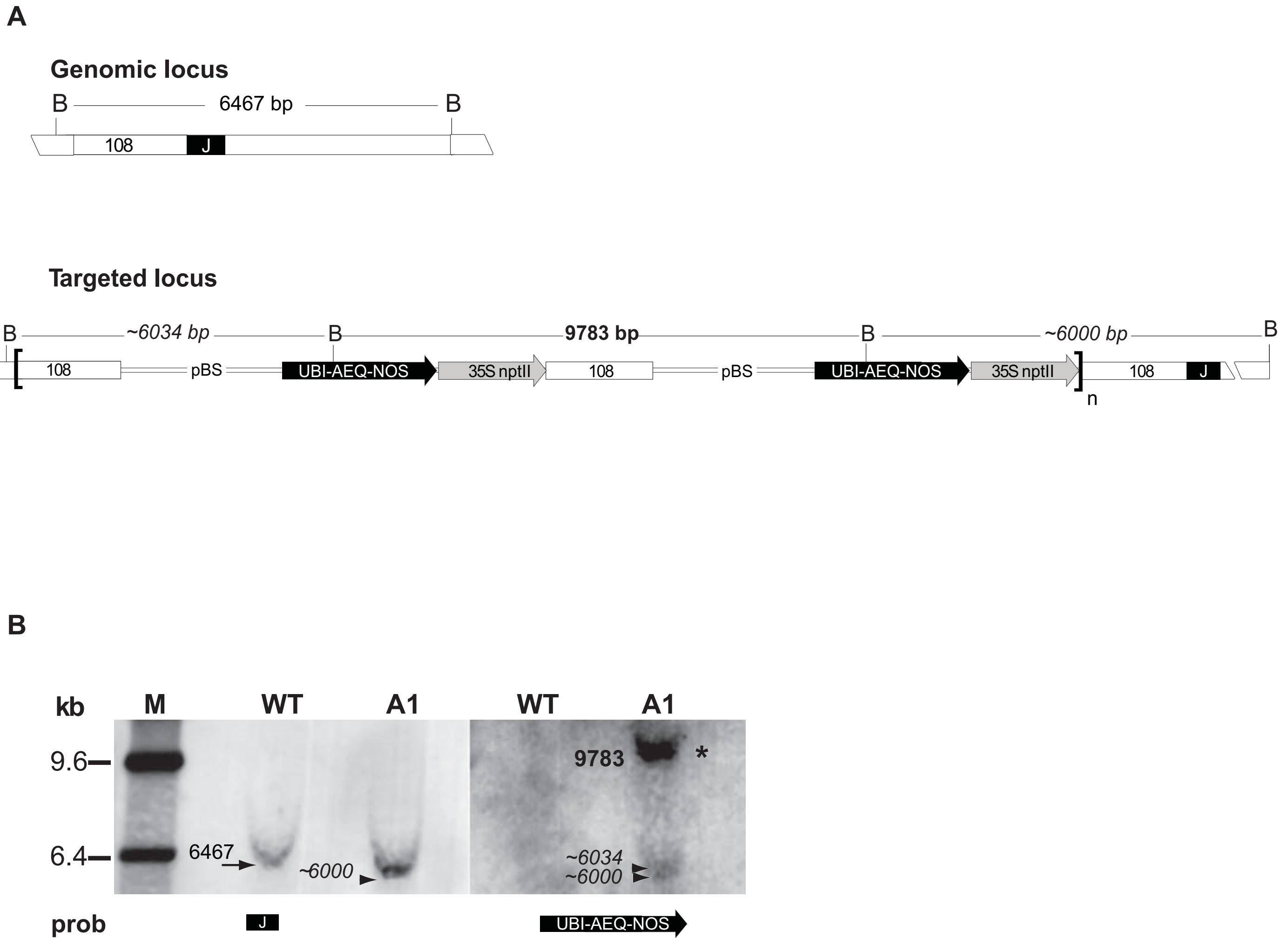


Supplemental Figure 7: Comparison of two independent UBI-AEQ lines. (A) Average lengths of apical protonemal cells measured in one week old colonies of wild type and two independent UBI-AEQ lines (A1 and A2). Mean + SD (n=20 cells for each strain). **(B)** Comparison of the responsiveness of UBI-AEQ lines to heat shock. A1 and A2 moss tissues were incubated at 40°C and $[Ca^{2+}]_{cyt}$ monitored every 3s during 18 minutes.



Supplemental Figure 8: Molecular analysis of UBI-AEQ moss line. (A) Predicted structure of targeted locus 108 following insertion by homologous recombination of two direct repeats of plasmid pBS108-II-UBI-AEQ. The wild-type and recombinant BglII (B) fragments are represented and their molecular weights are given in bp. The two probes used are: probe J of the 108 locus and probe UBI-AEQ-NOS. **(B)** Southern blot analysis of genomic DNA extracted from wild-type (WT) and UBI-AEQ (A1) plants. gDNA (10 µg) extracted from WT and A1 were digested with BglII and hybridized with probe J or probe UBI-AEQ-NOS. The regular number indicates the wild-type band (arrow); italicized numbers indicate the new hybrid junctions (arrow heads) and the bold number indicates direct repeats of the plasmid (*) (sizes are in bp).

Supplemental table 1

Twelve compounds, selected as calcium channel blockers, inhibitors of kinases or phospholipase C were tested in the *P. patens* HSP-GUS line for an inhibitory effect on the HSR.

	IC_{50} (µM)	References
Ca^{2+} channel blockers		
2-aminoethyldiphenyl borate (2-APB)	15	(Sandoval et al., 2007)
Flufenamic acid (FFA)	15	(Sandoval et al., 2007)
SKF-96365 hydrochloride	No inhibition	(Cabello and Schilling, 1993)
TMB-8 hydrochloride	No inhibition	(Bauer et al., 1999)
Ryanodine	No inhibition	(Sorrentino and Volpe, 1993)
Dantrolene sodium	No inhibition	(Ohta et al., 1990)
Phloretin	No inhibition	(Koh et al., 1994)
Amiloride hydrochloride	No inhibition	(Tang et al., 1988)
Kinase inhibitors		
Dicoumarol	10	(Seanor et al., 2003)
U1026	150	(Suri and Dhindsa, 2008)
PD 98059	No inhibition	(Suri and Dhindsa, 2008)
Phospholipase C inhibitor		
U-73122	30	(Liu et al., 2006)

HSP-GUS tissues were pre-treated at 22°C for 30 min with increasing concentrations of the above compounds, then heat-treated for 1h at 36°C and GUS activity was measured 8 hours post HS. The drop in Hsp-mediated GUS expression was measured and the IC_{50} determined.

SKF-96365: 1-{β-[3-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride.
TMB-8: 8-(N,N-Diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride.
U1026: 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene.
PD98059: 2′-Amino-3′-methoxyflavone.
U-73122: 1-{6-[(17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-1H-pyrrole-2,5-dione.

Supplemental references

B**auer, J., Dau, C., Cavarape, A., Schaefer, F., Ehmke, H., and Parekh, N.** (1999). ANG II- and TxA(2)-induced mesenteric vasoconstriction in rats is mediated by separate cell signaling pathways. American Journal of Physiology-Heart and Circulatory Physiology **277**, H1-H7.

Cabello, O. A., and Schilling, W. P. (1993). Vectorial Ca^{2+} Flux from the Extracellular-Space to the Endoplasmic-Reticulum Via a Restricted Cytoplasmic Compartment Regulates Inositol 1,4,5-Trisphosphate-Stimulated Ca2+ Release from Internal Stores in Vascular Endothelial-Cells. Biochemical Journal **295**, 357-366.

Koh, D. S., Reid, G., and Vogel, W. (1994). Activating Effect of the Flavonoid Phloretin on Ca2+-Activated K+ Channels in Myelinated Nerve-Fibers of Xenopus-Laevis (Vol 165, Pg 167, 1994). Neuroscience Letters **170**, 198-198.

Liu, H. T., Gao, F., Cui, S. J., Han, J. L., Sun, D. Y., and Zhou, R. G. (2006). Primary evidence for involvementof IP3 in heat-shock signal transduction in Arabidopsis. Cell Research **16**, 394-400.

Ohta, T., Ito, S., and Ohga, A. (1990). Inhibitory-Action of Dantrolene on Ca-Induced Ca^{2+} Release from Sarcoplasmic-Reticulum in Guinea-Pig Skeletal-Muscle. European Journal of Pharmacology **178**, 11-19.

Sandoval, A. J., Riquelme, J. P., Carretta, M. D., Hancke, J. L., Hidalgo, M. A., and Burgos, R. A. (2007). Store-operated calcium entry mediates intracellular alkalinization, ERK1/2, and Akt/PKB phosphorylation in bovine neutrophils. Journal of Leukocyte Biology **82**, 1266-1277.

Seanor, K.L., Cross, J.V., Nguyen, S.M., Yan, M., and Templeton, D.J. (2003). Reactive quinones differentially regulate SAPK/JNK and p38/mHOG stress kinases. Antioxid Redox Signal **5**, 103-113.

Sorrentino, V., and Volpe, P. (1993). Ryanodine Receptors - How Many, Where and Why. Trends in Pharmacological Sciences **14**, 98-103.

Suri, S. S., and Dhindsa, R. S. (2008). A heat-activated MAP kinase (HAMK) as a mediator of heat shock response in tobacco cells. Plant Cell and Environment **31**, 218-226.

Tang, C. M., Presser, F., and Morad, M. (1988). Amiloride Selectively Blocks the Low Threshold (T) Calcium-Channel. Science **240**, 213-215.

Supplemental table 2

Primers used to synthesize the probes for the detection of integrated pBS108-II-UBI-AEQ in the UBI-AEQ transgenic line A1.

Probe	template	Reference	Sense	Primer sequences
J	pGL108	Schaefer and Zryd, 1997	forward	GGACGCCTCTTGTTTTCTCTACATTC
			reverse	GGAAGTGGTGTGGTACGAGGTCATA
UBI-AEQ-NOS	pBS108-II-UBI-AEQ	This publication	forward	TTAACCCTCACTAAAGG
			reverse	CGATACTAGTGAATTCATCAGTGTTTTATT

Schaefer, D.G., and Zryd, J.P. (1997). Efficient gene targeting in the moss Physcomitrella patens. Plant Journal **11,** 1195-1206.

Supplemental Methods

Southern blot analysis

The moss genomic DNA was isolated from WT and transgenic line A1 and then digested by BglII (Promega). 10 µg were loaded and separated on 0.7% agarose gel followed by transfer to a nitrocellulose membrane (Hybond N^+; Amersham Pharmacia) using capillary transfer (Sambrook et al., 1989). DIG-labeled DNA probes were synthesized using the DIG DNA Labeling System (Roche), using primers and templates as indicated in Supplemental table 2. Detection was performed with the DIG Nucleic Acid Detection Kit (Roche, Rotkreutz, Switzerland) using anti-DIG–alkaline phosphatase conjugate and CDP-Star as a chemiluminescence substrate for alkaline phosphatase according to the manufacturer's instructions.