

Activation of the heat shock response in plants by chlorophenols: transgenic *Physcomitrella patens* as a sensitive biosensor for organic pollutants

YOUNOUSSE SAIDI¹, MARTA DOMINI^{2,3}, FLEUR CHOY³, JEAN-PIERRE ZRYD¹, JEAN-PAUL SCHWITZGUEBEL³ & PIERRE GOLOUBINOFF¹

¹Department of Plant Molecular Biology, Faculty of Biology and Medicine, University of Lausanne, CH-1015 Lausanne, Switzerland, ²Dipartimento di Ingegneria del Territorio, dell'Ambiente e delle Geotecnologie, Politecnico di Torino 10129, Torino, Italy and ³Laboratory for Environmental Biotechnology, Station 6, EPFL, CH-1015 Lausanne, Switzerland

ABSTRACT

The ability to detect early molecular responses to various chemicals is central to the understanding of biological impact of pollutants in a context of varying environmental cues. To monitor stress responses in a model plant, we used transgenic moss *Physcomitrella patens* expressing the β -glucuronidase reporter (GUS) under the control of the stress-inducible promoter *hsp17.3B*. Following exposure to pollutants from the dye and paper industry, GUS activity was measured by monitoring a fluorescent product.

Chlorophenols, heavy metals and sulphonated anthraquinones were found to specifically activate the *hsp17.3B* promoter (within hours) in correlation with long-term toxicity effects (within days). At mildly elevated physiological temperatures, the chemical activation of this promoter was strongly amplified, which considerably increased the sensitivity of the bioassay. Together with the activation of *hsp17.3B* promoter, chlorophenols induced endogenous chaperones that transiently protected a recombinant thermolabile luciferase (LUC) from severe heat denaturation. This sensitive bioassay provides an early warning molecular sensor to industrial pollutants under varying environments, in anticipation to long-term toxic effects in plants.

Because of the strong cross-talk between abiotic and chemical stresses that we find, this *P. patens* line is more likely to serve as a direct toxicity bioassay for pollutants combined with environmental cues, than as an indicator of absolute toxicity thresholds for various pollutants. It is also a powerful tool to study the role of heat shock proteins (HSPs) in plants exposed to combined chemical and environmental stresses.

Key-words: bioassay; chaperones; β -glucuronidase; *hsp* promoter; luciferase; moss; sulphonated anthraquinone.

Abbreviations: AQSA, anthraquinone-2-sulphonic acid; CP, DCP, TCP, PCP, mono-, di-, tri- and pentachlorophenol,

respectively; F_v/F_m , the maximal photochemical efficiency of PSII; GUS, β -glucuronidase; HS, heat shock; HSPs, heat shock proteins; LEA, late embryogenesis abundant protein; LUC, luciferase; MUG, 4-methylumbelliferyl- β -D-glucuronide; PSII, photosystem II.

INTRODUCTION

Human activity generates increasing amounts of new compounds that are released into the environment without prior knowledge of their potential toxicity or impact on living organisms. Chlorophenols are of industrial importance, and environmental concern as they are intermediates in the synthesis of various pesticides and are directly used as biocides and wood preservatives. Similarly, synthetic sulphonated aromatic compounds are the parent chemicals of a large array of dyes and detergents. Chlorinated and sulphonated aromatic compounds are recalcitrant to microbial degradation and can accumulate for years in soils or other environmental compartments (Czaplicka 2004). Moreover, they have acute and chronic effects on aquatic organisms (Greim *et al.* 1994; Schwitzguebel *et al.* 2002) and on human health (Czaplicka 2004).

A pollutant in the soil or in aquatic ecosystem can be detected by costly and time-consuming chemical analysis, which provides little information on the pollutant's toxicity to various organisms. Toxicity assessments often call for lengthy viability and fitness studies, with complex and contentious field experiments, involving large samples of populations. Another approach is to target the short-term molecular effects of a pollutant by monitoring the cellular reactions to chemical aggression. This can be achieved by focusing on the molecular responses to natural stresses, such as HS, leading to the build-up of particular defence mechanisms, such as the accumulation of molecular chaperones. Conceptually, when exposed to a toxic chemical compound, all living organisms must rapidly induce some sort of molecular reaction to set up cellular defences, which, when successful, can lead to adaptation. Yet, when such defence mechanisms fail to fully protect the organism, toxic effects may prevail and lead to growth arrest, infertility and ultimately to lethality. Thus, the sensitivity and specificity of

Correspondence: P. Goloubinoff. Fax: +41 21 692 4195; e-mail: pierre.goloubinoff@unil.ch

a biosensor to a given toxic agent depend on the type of molecular responses that are naturally elicited and that can be recruited in its design.

Most of the existing biosensors are based either on a promoter/operator fused to a reporter gene or on a constitutively expressed sensitive reporter protein (Sorensen, Burmolle & Hansen 2006). There are numerous microbial- (Sorensen *et al.* 2006; Tecon & van der Meer 2006), yeast- (Jayaraman *et al.* 2005; Schmitt, Gellert & Lichtenberg-Frate 2005), nematode- (Candido & Jones 1996; Lagido *et al.* 2001) and mammalian- (Ait-Aissa *et al.* 2000) based biosensors. However, examples of plant biosensors are significantly fewer (Meers *et al.* 2006). Although sensitive, bacterial biosensors do not necessarily allow the extrapolation of data to eukaryotic multicellular organisms. Moreover, the toxic mechanism of some compounds may be based on their interaction with specific eukaryotic proteins, such as the aryl hydrocarbon receptor (Hillegass *et al.* 2006), and cannot be addressed simply when using bacteria. Plants can be used for the phytoremediation of soils. To this aim, knowledge is needed about the effect of pollutants on plant cells, which can be provided only partially by bacterial, yeast or nematode biosensors. Thus, there is a growing need for a simple and sensitive plant-based biosensor that can readily provide information about the molecular effects of pollutants, their potential toxicity and the consequences of long-term exposures.

As sessile organisms, plants have developed powerful defence mechanisms to cope with biological and environmental aggressions (Vinocur & Altman 2005). They can rapidly respond to small variations in their environment by the synthesis of stress proteins, in anticipation to more damaging conditions. The molecular chaperones are defence proteins that are induced to prevent and cure protein aggregation (Wang *et al.* 2004; Hinault, Ben-Zvi & Goloubinoff 2006). The small HSPs (sHSPs) are molecular chaperones containing an α -crystalline-like domain (Van Montfort, Slingsby & Vierling 2001), which are rapidly and massively induced to bind heat- or mutation-induced misfolding proteins and can also stabilize membranes during abiotic stresses. HSPs thus prevent both stress-induced protein aggregation and membrane damages (Veinger *et al.* 1998; Torok *et al.* 2001). In many eukaryotes, a small non-stressful temperature rise suffices to induce the synthesis of sHSPs under conditions where the synthesis of other molecular chaperones is not yet observed (Boston, Viitanen & Vierling 1996). Several members of the sHSPs family are also induced in plants by oxidative, osmotic and heavy metal stresses (Adamska, Ohad & Klopstsch 1992; Sun, Van Montagu & Verbruggen 2002). Therefore, to design a sensitive heat- and pollutant-specific biosensor in the moss *Physcomitrella patens*, we chose to use the promoter of a soybean sHSP (Saidi *et al.* 2005).

Physcomitrella patens is a small land plant, which is highly amenable to genetic manipulations (Schaefer & Zryd 2001; Cove 2005). Morphologically, this moss is particularly appropriate for biomonitoring studies because during the first stage of its development, it grows as single cell-wide

filaments 'protonemata' that can easily be cultured on agar plates or in liquid medium. The ability of *P. patens* to withstand complete immersion is optimal for toxicity analysis: it allows uniform exposure of all moss cells to the tested chemicals. Moreover, in contrast to vascular plants, *P. patens* lacks cuticles, hydrophobic lignin and suberin barriers. Thus, chemical compounds do not need to cross an endodermis barrier, and do not need to be transported by the xylem to reach all the plant cells, as it is the case with seed-plants like *Arabidopsis thaliana*.

We have initially developed a bioassay for mild changes of temperature with the transgenic *P. patens* line HSP-GUS (Saidi *et al.* 2005). This line contains a *uidA* reporter gene encoding GUS enzyme under the control of the soybean heat-inducible promoter *hsp17.3B*. In *P. patens*, this promoter was found to be virtually non-leaky at 25 °C allowing detection of molecular responses following small changes of temperature (Saidi *et al.* 2005).

Here, using this HSP-GUS line, we have developed a simple screen to address the effect of various pollutants generated by the textile and wood industry, as potential elicitors of the HS-like response in plants. We found that several common pollutants can significantly induce the HS response in moss cells in correlation with subsequent long-term toxicity. This new type of plant biosensor is therefore useful to detect and characterize the effect of specific toxic pollutants under varying environmental conditions.

MATERIALS AND METHODS

Plasmids, plant material and growth conditions

Physcomitrella patens (Gransden wild type) was grown on an agar minimal medium (Ashton, Grimsley & Cove 1979) overlaid with a cellophane disk and transferred when necessary to a liquid medium. Culture, growth and transformation were performed as described earlier (Schaefer & Zryd 1997). The HSP-GUS and the LEA-GUS lines were described in Saidi *et al.* (2005) and Kamisugi & Cuming (2005), respectively. The 35S-LUC line was generated as follows: *P. patens* protoplasts were transformed with 10 µg of p35S-LUC-MU108 vector. This vector was generated by blunt cloning a 2.5 Kb fragment containing the 35S promoter, the firefly LUC cDNA and the ACMV terminator in the *HindIII*-digested targeting vector, carrying a hygromycin resistance cassette driven by the 35S promoter in addition to the PP-108 targeting sequence (Schaefer & Zryd 1997).

Heat and chemical treatments

One-week-old HSP-GUS, 35S-LUC or LEA-GUS protonemal tissues were transferred to a liquid minimal medium in multiwell plates (Costar, Corning Incorporated, Corning, NY, USA) and heat or chemically treated in a temperature-controlled chamber at indicated temperatures and durations. Chemicals were from Merck (Rahway, NJ, USA) [AQSA, toluene-4-sulphonic acid (TOL)], Fluka (Milwaukee, WI, USA) [benzene-sulphonic acid (BNZ),

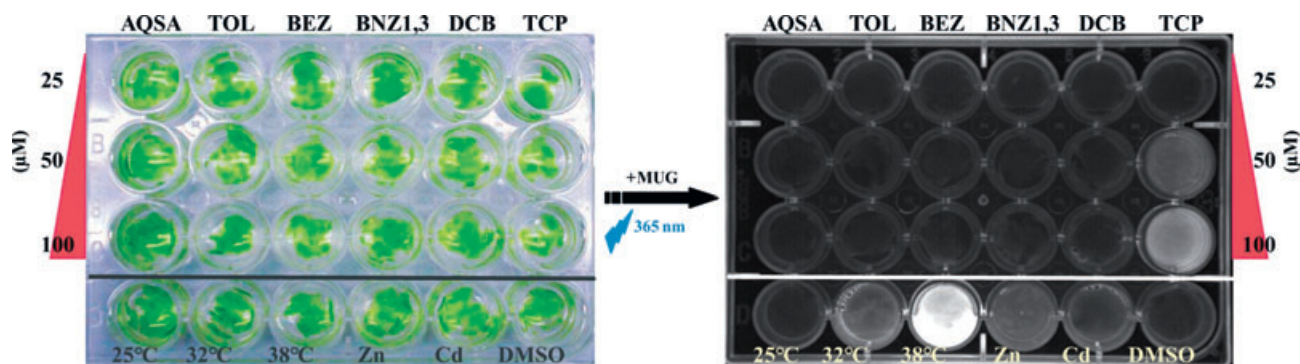


Figure 1. Screening of sulphonated and chlorinated aromatic compounds for activation of the *hsp17.3B* promoter. HSP–GUS protonemal cells were treated for 20 h at 25 °C in liquid medium supplemented with indicated concentrations of different compounds. Protonemata were then washed and cultured in a new liquid medium for an additional 16 h. After the addition of MUG substrate, tissues were incubated at 37 °C and fluorescence monitored at 365 nm wavelength (right multiwell). The compounds tested were AQSA, toluene-4-sulphonic acid (TOL), benzene-sulphonic acid (BNZ), benzene-1,3-disulphonic acid (BNZ1,3), 1,2-dichlorobenzene (DCB) and 2,4,6-TCP. The bottom row of the multiwell carries tissues treated in liquid medium supplemented with zinc dichloride (20 h, 100 μM), cadmium dichloride (20 h, 100 μM), dimethyl sulphoxide (DMSO) (20 h, 0.1%) or in chemical-free medium at 25, 32 or 38 °C for 1 h.

benzene-1,3-disulphonic acid (BNZ1,3), *O*-acetylsalicylic acid (ASA), benzyl alcohol, sodium salicylate] and Sigma (St Louis, MO, USA) (all chlorophenols). Celastrol was kindly provided by R.I. Morimoto (Northwestern University). All chemicals were dissolved in sterile water except for AQSA, *O*-ASA, celastrol and chlorophenols, which were dissolved in dimethylsulphoxide (DMSO). The final DMSO concentration never exceeded 0.1%, which has no effect on GUS expression (Saidi *et al.* 2005). Unless otherwise stated, heat or chemically treated mosses were tested for GUS activity following 16 h of recovery at 25 °C.

GUS assay

For the semi-quantitative test (Fig. 1), 1-week-old HSP–GUS protonemal cells were treated in multiwell plates for 20 h at 25 °C in a liquid medium supplemented with indicated concentrations of different compounds. Tissues were then washed three times with the minimal medium, and cultured in a new liquid medium for 16 h. After permeabilization and fixation with a solution containing 0.3% (v/v) formalin, 5.45% (w/v) mannitol and 0.2% (w/v) 2-(*N*-Morpholino)ethanesulphonic acid (MES)–KOH (pH 5.6) for 30 min, tissues were washed three times with 50 mM NaPO₄ (pH 7.0). The MUG substrate (2 mM) was then added, and tissues were incubated at 37 °C for 60 min. Tissues were observed under UV lamp (365 nm), and pictures were taken using a digital camera. For quantitative measurements, the GUS activity was determined according to Jefferson, Kavanagh & Bevan (1987), using 2 mM of MUG as a substrate (Saidi *et al.* 2005). Protein concentrations in extracts were determined by the Bradford method (Bio-Rad, Hercules, CA, USA). The GUS activity was expressed in nmol MU produced per minute per milligram of protein.

LUC assay

For *in vivo* thermo-protection measurements, 35S–LUC tissues were pre-treated with 100 μM 2,4,6-TCP during 4 h

at 25 °C or at 32 °C for 1 h. Following 6 h recovery at 25 °C, thermal inactivation of LUC was performed at 42 °C during 15 min using heating block. During heat inactivation, aliquots were taken every minute and frozen in liquid nitrogen. Total soluble proteins were then extracted at 4 °C using CCLR extraction buffer (Promega, Madison, WI, USA), and 5 μL of each extract was loaded in a microtitre plate. The LUC activity was measured in a Perkin Elmer (Wellesley, MA, USA) microtitre plate luminescence counter (1420 VICTOR light) after injection of luciferin substrate (Promega). Protein concentrations in extracts were determined by the Bradford method. LUC activities were expressed as count per second per milligram of protein, and were converted as percentage of the activity before inactivation at 42 °C.

Western blotting

For Western blot analysis, 15 μg of moss crude soluble protein extracts was fractioned on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting, and incubated with a rabbit-derived polyclonal antibody against HSP90 (Stress-Gen, Ann Arbor, MI, USA) (1/35 000, v/v) or against GUS protein (Molecular Probes, Eugene, OR, USA) (1/500, v/v). The blots were then incubated with HRP-conjugated anti-rabbit IgG (Promega) diluted 1/15 000 (v/v). Immune complexes were visualized using the chemiluminescent Immunstar Kit (Bio-Rad) according to the manufacturer's instructions.

Measurement of PSII activity

Chlorophyll *a* fluorescence measurements were made with the plant efficiency analyser (PEA) from Hansatech Instruments Ltd (Norfolk, England). The photosynthetic activity

was measured in moss tissues after dark adaptation of 2 min as detailed in Saidi *et al.* (2005).

Viability test, live cell microscopy and image analysis

For cell viability estimation, 6-carboxyfluorescein diacetate (Fluka) stock solution of 5 mg in 1 mL acetone was prepared and then added to moss tissues at final concentration of 1 mg mL⁻¹. After 2–5 min at room temperature, observations were performed under a microscope with fluorescence filter (absorption: 490 nm; emission: 514 nm). Pictures were taken using a LEICA DC 200 camera (Leica Microsystems, Heerbrugg, Switzerland). Fluorescence and white light images were merged and processed using Photoshop 6.0 software (Adobe Systems).

RESULTS

Screening for aromatic compounds that activate the HS response in *P. patens*

The stable HSP–GUS moss line was used in a semi-quantitative screen to test the effect of aromatic pollutants that may induce an HS response. Figure 1 shows a fast, simple GUS assay on whole plant tissues. Using a multiwell plate, mats of moss protonemata were treated for 20 h in a

liquid medium containing different concentrations of the indicated pollutants, and compared to heat-treated controls. Sixteen hours after removal of the pollutants, the GUS activity in fixed tissues was monitored by the appearance of the fluorescent product of GUS reaction.

Whereas at 25 °C, most of the organic pollutants tested (up to 100 µM) did not elicit any observable (Fig. 1) or measurable (Table 1) GUS activity; exposure to relatively low concentrations of 2,4,6-TCP induced significant GUS activity (Fig. 1; Table 1). One hour exposure to TCP (100 µM) sufficed to induce GUS expression 10-fold higher than in the untreated control (Fig. 2a). Increasing the exposure time to 4 or 20 h resulted in a respective 30- or 50-fold increase of GUS expression (Fig. 2a). Beyond 20 h exposure, GUS expression decreased (data not shown) likely because of cell death or lower rates of protein synthesis. At equal concentration (100 µM), 2,4-DCP or 2,6-DCP were both about seven times less potent, and 2-chlorophenol or 4-chlorophenol (CP) about 10 times less potent elicitors than TCP (Fig. 2a; Table 1). PCP was the most powerful elicitor of the HS-like response: at constant time of exposure, 2 µM PCP sufficed to induce a significant GUS expression, similar to the effect of 20 µM TCP (Fig. 2b). The induced GUS expression increased with the concentration of the elicitor (Fig. 2b,c): maximal fold induction was reached upon exposure to 10 µM PCP or 100 µM TCP. It can

Compound/temperature	Treatment duration (h)		
	1	4	20
25 °C	0.88 ± 0.32	0.66 ± 0.14	0.71 ± 0.45
Dimethylsulphoxide (DMSO) (0.1%)	0.79 ± 0.39	0.51 ± 0.86	0.82 ± 0.31
AQSA (100 µM)	–	1.02 ± 0.32	1.13 ± 0.41
Toluene-4-sulphonic acid (TOL) (100 µM)	–	0.77 ± 0.47	0.75 ± 0.26
Benzene-sulphonic acid (BNZ) (100 µM)	–	0.86 ± 0.52	0.80 ± 0.31
Benzene-1,3-disulphonic acid (BNZ1,3) (100 µM)	–	0.82 ± 0.31	0.75 ± 0.52
1,2-dichlorobenzene (DCB) (100 µM)	–	0.80 ± 0.22	0.73 ± 0.31
PCP (10 µM)	11.21 ± 2.87	29.2 ± 6.53	–
2,4,6-TCP (100 µM)	8.47 ± 3.10	27.5 ± 8.30	60.3 ± 21.2
2,4-DCP (100 µM)	–	4.17 ± 0.16	8.51 ± 0.86
2,6-DCP (100 µM)	–	3.47 ± 0.52	7.92 ± 1.11
2-CP (100 µM)	–	2.47 ± 0.75	5.63 ± 1.61
4-CP (100 µM)	–	2.37 ± 0.21	6.59 ± 1.06
Phenol (100 µM)	–	0.81 ± 0.42	0.74 ± 0.41
Cadmium dichloride (100 µM)	–	2.21 ± 0.56	3.72 ± 0.23
Zinc dichloride (100 µM)	–	4.07 ± 0.94	5.97 ± 0.42
Benzyl alcohol (30 mM)	32.5 ± 3.67	–	–
Acetylsalicylic acid (ASA) (0.8 mM)	56.9 ± 10.7	–	–
Sodium salicylate (4 mM)	18.2 ± 3.87	–	–
Sodium salicylate (10 mM)	85.3 ± 8.71	–	–
Celastrol (4 µM)	64.6 ± 15.7	–	–
Celastrol (12 µM)	100 ± 13.7	–	–
32 °C	30.5 ± 7.81	–	–
38 °C	870 ± 25.3	–	–

Moss tissues were exposed to different organic compounds or HS.

GUS specific activities were measured from three independent experiments following 16 h recovery after each treatment.

Standard deviations are shown. Activities are given in nmol MU min⁻¹ mg⁻¹ protein. Significant GUS inductions are shown in boldface.

Table 1. GUS specific activities in HSP–GUS line extracts after screening for the activation of *hsp17.3B* stress promoter

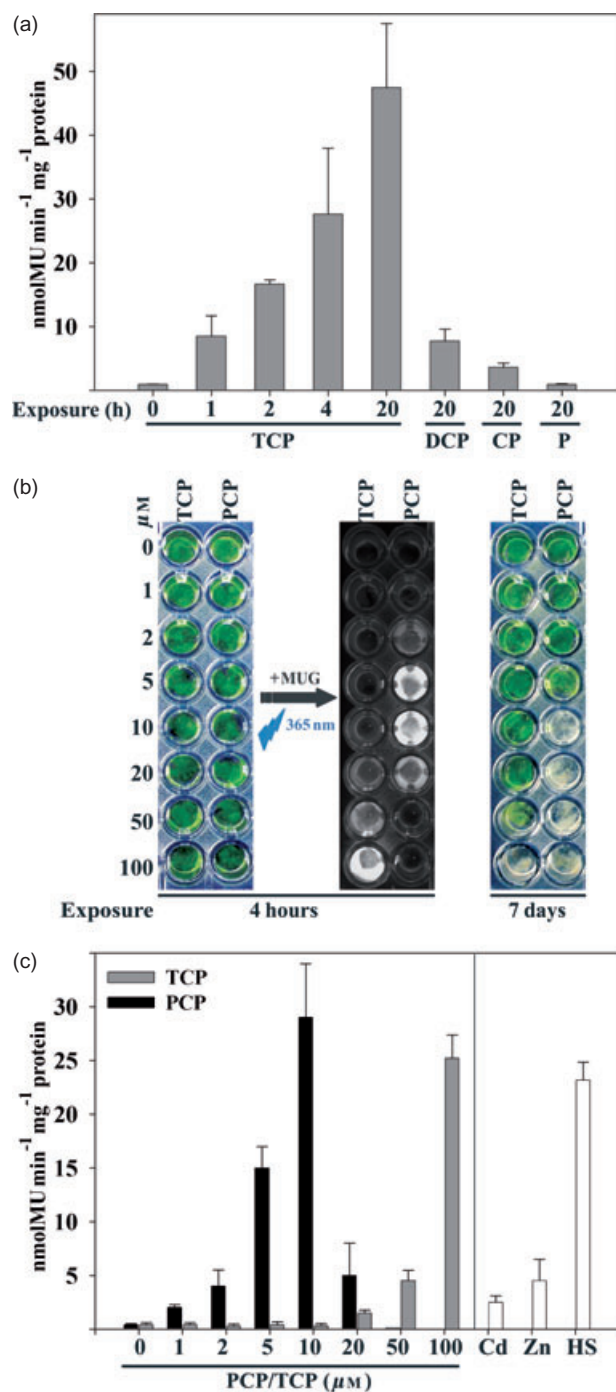


Figure 2. The *hsp17.3B* promoter is activated by chlorophenols in a dose-dependent manner. (a) Effect of increasing times of exposure to chlorophenols. HSP-GUS tissues were exposed in liquid medium to 100 μ M TCP, 2,4-DCP, 2-chlorophenol (CP) or phenol (P). (b) Exposure to increasing concentrations of PCP or TCP in a 96-multiwell as in Fig. 1. (c) GUS specific activities measured in samples from (b, 4 h) as well as from tissues exposed to zinc dichloride (4 h, 100 μ M), cadmium dichloride (4 h, 100 μ M) or HS (1 h, 32 °C). GUS specific activities were measured after 16 h of recovery. Values are means of three independent experiments, and standard deviations are shown.

be concluded that the activation of the HS promoter at non-inducing temperature depends on the number of chlorine atoms in the chlorophenol molecule.

Confirming that heavy metals can induce HSPs in eukaryotes (Hall 2002; Uenishi *et al.* 2006), exposure to 100 μ M cadmium or zinc also induced a significant GUS expression (Figs 1 & 2c). This indicates that the HSP-GUS biosensor can also be used to detect heavy metal pollutants.

The *hsp17.3B* promoter is specifically activated by temperature and chlorophenols

The endogenous promoter for a LEA protein of *P. patens* (*PpLEA-1*) is naturally activated by osmotic stress or by the addition of abscisic acid (ABA) (Kamisugi & Cuming 2005). Here, we used the LEA-GUS line, expressing GUS from the *PpLEA-1* promoter, to investigate the specificity of the *hsp17.3B* promoter in reaction to various abiotic and chemical treatments. As previously shown (Kamisugi & Cuming 2005), the LEA promoter was significantly activated by treatments with NaCl (0.3 M), mannitol (10%) and especially by ABA (100 μ M). Remarkably, neither TCP (100 μ M), ASA (0.8 mM) nor HS (34 °C) were effective to induce GUS expression in this line (Fig. 3). Conversely, there was no activation of the HSP promoter in the HSP-GUS line exposed to osmotic stress or ABA treatment, although TCP, ASA or HS showed strong GUS activation. Therefore, the response of the recombinant *hsp17.3B* promoter to chlorophenols is highly specific.

Short exposure to TCP induces *in vivo* protection of thermolabile LUC

To test the physiological short-term effects of chlorophenol treatment on the induction of the whole chaperone network

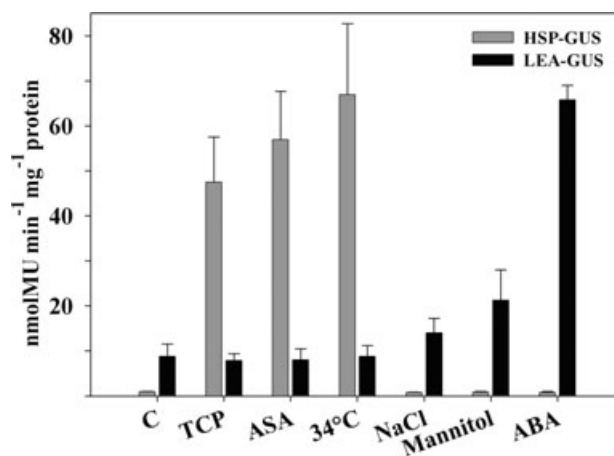


Figure 3. The HS promoter is specifically activated by chlorophenols. LEA-GUS or HSP-GUS tissues were treated for 20 h at 25 °C in liquid medium (c) or in liquid medium supplemented with TCP (100 μ M), sodium chloride (0.3 M), mannitol (10%) or abscisic acid (ABA) (100 μ M). Moss cells were also exposed 1 h to acetylsalicylic acid (ASA) (0.8 mM) at 25 °C or treated 1 h at 34 °C. GUS specific activities were measured after 16 h of recovery. Values are means of three independent experiments, and standard deviations are shown.

in chemically stressed plant cells, we used a 35S-LUC moss line that constitutively expresses firefly LUC from the 35S promoter. Firefly LUC is a thermolabile recombinant protein, which denatures *in vivo* during short HS treatments non-damaging to the host cell (Fig. 4a). The 35S-LUC tissues were first pre-treated either at 25 °C with TCP (100 μ M, 4 h) or at 32 °C (1 h), followed by 6 h recovery at 25 °C to allow synthesis and accumulation of stress-induced HSPs. Then, moss tissues were exposed to 42 °C for 14 min, and the *in vivo* thermal inactivation rates of LUC were followed (Fig. 4b). In the untreated tissues, LUC half-life at 42 °C was about 2 min. In contrast, in the 32 °C- or TCP-pre-treated tissues, the inactivation half-life values were 7 and 9 min, respectively (Fig. 4b). *In vitro* analysis showed that high TCP concentrations have no effect on the rate of LUC heat inactivation (data not shown). This indicates that an enforced HSP network is induced in TCP pre-treated samples, which can protect and reactivate some of the denatured LUC during the severe stress, thus slowing down the apparent rate of inactivation. Western blot analysis confirmed a significant concomitant accumulation of Hsp90 chaperones and of GUS at 32 °C and in TCP-pre-treated tissues but not in untreated controls (Fig. 4c). The fact that TCP can induce a futile accumulation of the chaperone network suggests a long-term cost that can affect the plant fitness and viability.

Effect of chlorophenols on plant fitness and cell viability

We next explored the correlation between short exposures to TCP or PCP, and long-term damages and cell death, as it is the case with prolonged excessive heat stress. Two complementing approaches were used to estimate the physiological well-being of the moss: the photosynthetic activity by measuring the F_v/F_m ratio (Strasser, Srivastava & Govindjee 1995) and the cell viability using the dye fluorescein diacetate (FDA), which labels only living cells (Lee *et al.* 2004).

A 20 h exposure to increasing concentrations of TCP displayed a dose-dependent decrease of the F_v/F_m ratio measured immediately after the treatment. Noticeably, whereas the effect of 100 μ M TCP on the photosynthetic activity was fully recovered 16 h after TCP removal, the effect of 100 μ M PCP was more severe and irreversible (Fig. 5a). When the effects of PCP, TCP, DCP and CP were compared, the decrease of F_v/F_m ratio correlated with the increasing number of chlorine atoms in the chlorophenol molecule (data not shown). Viability tests were performed following 1 and 3 d exposure to either TCP or PCP. Whereas significant cell death was observed already following 1 d exposure to 100 μ M PCP, 3 d were required to observe the same lethal effect with 100 μ M TCP (Fig. 5b). Additionally, a good correlation was found between the extent of the HS-like molecular response in the short term (hours) and the extent of damages affecting plant survival in the long term (days) (Fig. 2b). Similar toxic effects were also

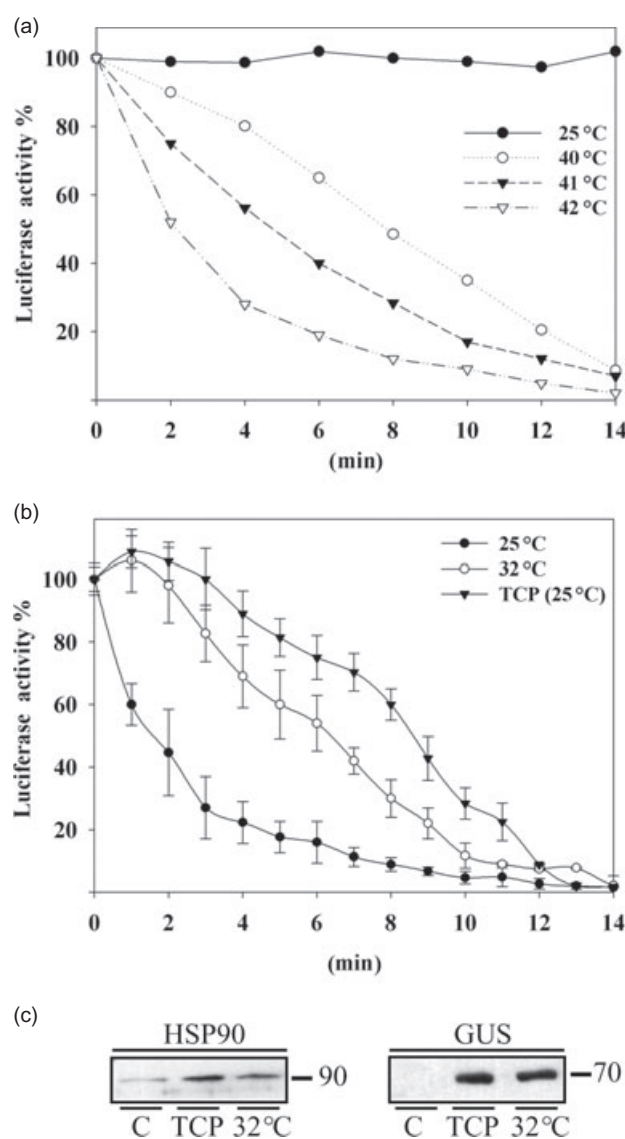


Figure 4. Chlorophenols induce chaperone network that protects thermolabile LUC. (a) *In vivo* LUC thermal inactivation at different temperatures. Protonemal tissues from 35S-LUC were incubated at 25, 40, 41 and 42 °C for 14 min. LUC activity was measured every 2 min and expressed relatively to that before incubation, which was taken as 100%. (b) *In vivo* LUC thermal inactivation at 42 °C following different pre-treatments. Six hours before the incubation at 42 °C, 35S-LUC tissues were pre-treated 1 h at 32 °C (open circles) or 4 h at 25 °C with 100 μ M TCP (closed triangles). During the thermal inactivation, LUC activity was monitored every minute and expressed relatively to the initial value at time 0 (set as 100%). The inactivation rates were then compared with that of non-treated tissues (closed circles). (c) Immunoblot analysis of cytosolic HSP90 amounts using total protein extracts isolated from the non-treated (C), TCP (25 °C) or heat-treated (32 °C) tissues immediately before thermal inactivation experiment (point 0 in Fig. 4b). GUS amounts in extracts from HSP-GUS line were also determined after similar treatments.

observed following prolonged exposures to cadmium and zinc dichloride (data not shown).

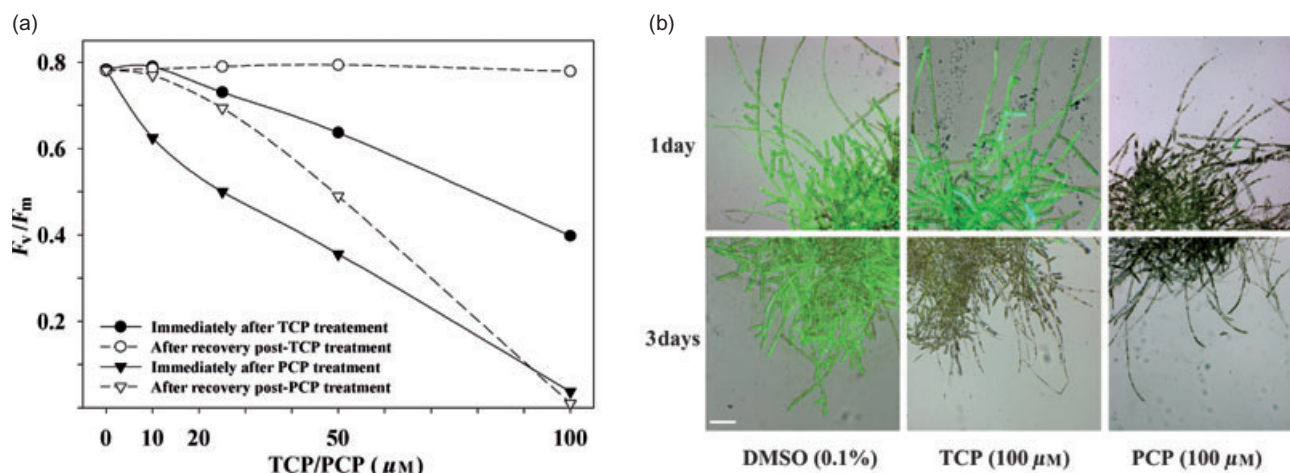


Figure 5. Chlorophenol affects plant fitness and cell viability. (a) Effect of TCP or PCP on the photosynthetic activity. The F_v/F_m was measured in tissues immediately after 20 h exposure to different concentrations of TCP (closed circles) or PCP (closed triangles), and in the end of 16 h of recovery (open circles and triangles). (b) Viability test based on fluorescein diacetate (FDA). Moss tissues were exposed to indicated concentrations of TCP, PCP or dimethylsulphoxide (DMSO) for 1 or 3 d. Immediately after the treatment, FDA was added and the number of fluorescent-viable cells was estimated under fluorescent microscope. Pictures display merged white light and fluorescence images. Bar: 200 μm .

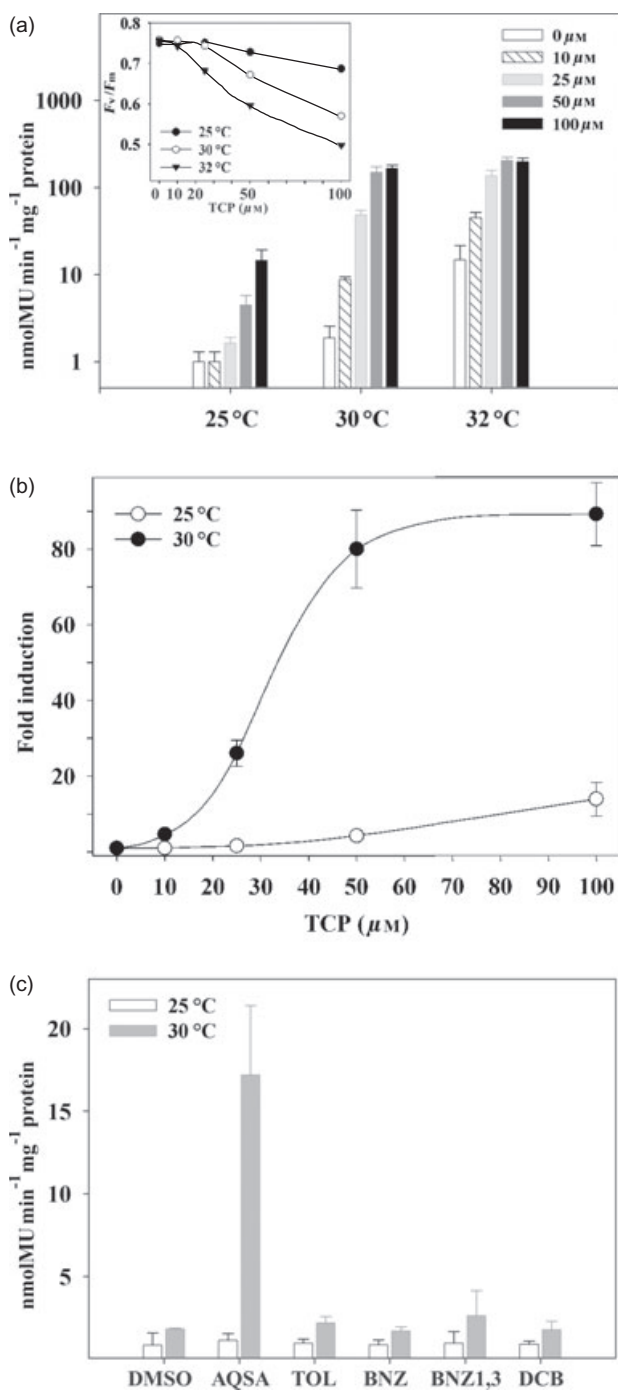
Physiological mild HS enhances the pollutant effects

It has been previously shown that induction of HS genes by membrane-interacting compounds, such as benzyl alcohol and bimocmolol, can be strongly improved when exposures are performed at elevated, albeit non-damaging, temperatures (Hargitai *et al.* 2003; Torok *et al.* 2003). Here, we compared the effects of increasing concentrations of TCP at 25, 30 or 32 °C, which are all physiological temperatures with no apparent effects on the moss physiology (Saidi *et al.* 2005). A concomitant 1 h exposure to increasing TCP concentrations resulted in a strong amplification of the GUS induction at 30 and 32 °C, as compared to 25 °C (Fig. 6a). Whereas at 25 °C, 10 μM TCP had no effect; at 30 °C, the GUS induction by the same concentration was fivefold higher than without TCP. Similarly, whereas at 25 °C, 25 μM TCP barely induced GUS expression (1.6-fold), at 30 °C the GUS induction by the same concentration was 26-fold higher than without TCP (Fig. 6a). Thus, when tested at slightly higher, but still physiological temperatures, the plant bio-sensing assay became remarkably more sensitive to chlorophenol. Whereas at 25 °C, 40 μM TCP was needed to reach a significant twofold induction threshold; at 30 °C, this was reduced to 4 μM TCP, corresponding to a 10-fold increase in the sensitivity of the HSP-GUS line to the pollutant (Fig. 6b). By measuring the F_v/F_m ratio, a tight correlation was observed between the synergistic GUS expression and the decrease of photosynthetic activity above 10 μM TCP. At 30 °C, no detectable effect on photosynthetic activity was observed up to 25 μM TCP, although a significant activation of the stress promoter was recorded. This indicates that the molecular assay based on the *hsp17.3B* promoter is more sensitive than standard fitness and long-term physiological assays.

Confirming the importance of increasing the sensitivity of the bioassay by mild elevated temperature, 1 h exposure to AQSA (100 μM) was found to induce a significant GUS signal at 30 °C, which was not observed at 25 °C (Fig. 6c). This activation of the *hsp17.3B* promoter suggests a long-term toxic effect on moss physiology.

DISCUSSION

In this study, a recombinant *P. patens* moss line, HSP-GUS, was used as biosensor for stress and toxicity assessments in plant tissues. This line was previously shown to strongly react to temperature changes and also to some chemicals, such as benzyl alcohol and acetylsalicylic acid (Saidi *et al.* 2005), suggesting that it can serve as a sensitive phyto-bioassay to potential organic pollutants. The GUS reporter is a highly stable protein that can withstand harsh denaturing conditions *in vivo*. We reasoned that unwanted direct destabilizing effects of the tested compounds on the reporter protein may be less significant in a thermostable enzyme, such as GUS, than in a thermolabile enzyme, such as LUC (Jefferson *et al.* 1987; Forreiter, Kirschner & Nover 1997). In moss cells, GUS has a half-life of 4 d at 25 °C (Saidi *et al.* 2005). This is most appropriate for stress bio-sensing assays aiming at detecting molecular responses within the first hours of a chemical stress, as well as days after the treatment (when toxicity starts to appear). In addition, with this system, weak signals can be amplified *in vivo* by reiteration of the inducing treatment, and *in vitro* by prolonging the incubation of the GUS enzyme with its substrate. Such *in vitro* enhancement of the signal is not possible with fluorescent reporter proteins.



Chlorophenols and sulphonated anthraquinones as inducers of HS response

Among the several pollutants tested here, only chlorophenols clearly showed a strong and specific activation of the *hsp17.3B* promoter (Table 1). The short-term molecular effects of chlorophenols, which were proportional to the degree of chlorination and to the duration of the exposure, correlated well with long-term toxicity (Figs 2 & 5). These data confirmed earlier toxicity reports for chlorophenols in algae, *Daphnia*, fishes and humans (Shigeoka *et al.* 1988;

Figure 6. Sensitivity of HSP-GUS increased at physiological elevated temperatures. (a) HSP-GUS protonemal tissues were exposed to indicated concentrations of TCP during 1 h at 25, 30 or 32 °C. Inset, F_v/F_m measured immediately after treatments as in (a). (b) Relative GUS activities from (a) expressed as fold induction of the expression level in (0 μM) at respective temperatures. (c) Screening of non-eliciting compounds for co-activation of the *hsp17.3B* promoter at 30 °C. HSP-GUS cells were exposed 1 h at 25 or 30 °C to 100 μM AQSA, toluene-4-sulphonic acid (TOL), benzene-sulphonic acid (BNZ), benzene-1,3-disulphonic acid (BNZ1,3), 1,2-dichlorobenzene (DCB) or to 0.1% dimethylsulphoxide (DMSO) in which AQSA was dissolved. Prior to estimation of GUS specific activities, moss cells were washed and incubated 16 h in a new medium at 25 °C. Values are means of three independent experiments, and standard deviations are shown.

Harvey *et al.* 2002; Czaplicka 2004). Here, we showed that simple tests of GUS activity in a small sample of moss cells, following short exposures to low concentrations of different pollutants, can provide a sensitive and rapid indication about the potential toxicity of new organic compounds. The sensitivity of the plant *hsp17.3B* promoter was comparable to that of the human *hsp70* promoter, which can be activated by the same range of chlorophenol concentrations in HeLa cells (Ait-Aissa *et al.* 2000). This suggests that plants react to this class of toxic pollutants by a similar mechanism as in mammalian cells.

With regard to sulphonated anthraquinone, a significant GUS expression was observed at 30 °C within 1 h of exposure to 100 μM AQSA. Such GUS induction remained unclear at 25 °C even after 20 h exposure (Table 1). The AQSA-mediated activation of the *hsp17.3B* promoter was specific because no effect of this compound on the LEA promoter was observed (data not shown). There is thus far no information on the type of molecular damages that may be caused by AQSA to plant cells. Our observation of a weak triggering of the HS response in the moss suggests that AQSA can be toxic under given conditions. Indeed, after 7 d of continuous exposure to 200 μM AQSA at 32 °C, but not with lower concentrations, we observed dramatic effects on cell viability (data not shown). Hence, *P. patens* may adapt to sulphonated anthraquinone at concentrations lesser than 200 μM, in agreement with previous reports that some plants, such as rhubarb, can accumulate and metabolize several sulphonated aromatic compounds (Duc *et al.* 1999; Schwitzguebel *et al.* 2002; Aubert & Schwitzguebel 2004).

Other pollutants tested [TOL, BNZ, BNZ1,3 and 1,2-dichlorobenzene (DCB)] showed no significant induction of the HS response (Fig. 6c, Table 1). Additional analysis revealed no effects of these compounds on plant fitness or on cell viability (data not shown). Future research may include testing the combined effects of mixtures of pollutants, also using moss lines expressing GUS under the control of other stress-inducible promoters.

The use of a constitutively expressed thermolabile reporter protein, LUC, offered an insight on the activity of the chaperone network in response to aggression by toxic

pollutants. A short pre-conditioning with TCP, like 32 °C pre-treatment, induced an *in vivo* thermo-protection of the LUC at 42 °C (Fig. 4b). During such a short HS (14 min), there is no significant synthesis of neither chaperones (Saidi *et al.* 2005) nor LUC. The apparent thermo-protection was most likely caused by endogenous chaperones and sHSPs that were induced during the pre-conditioning phase and thus reactivate and protect some of the heat-denaturing LUC. Hence, chlorophenol activation of the recombinant *hsp17.3B* promoter strongly suggests that a general HS response has occurred.

Possible mechanisms for chlorophenol-induced HS response

It has been previously suggested that chlorophenols act as uncoupling agents that destroy the electrochemical proton gradient across membranes of *Acer* cell suspensions in the photosynthetic bacterium *Rhodobacter sphaeroides* (Ravanel, Taillandier & Tissut 1989; Escher, Snozzi & Schwarzenbach 1996). Yet, uncoupling agents have also been shown to inhibit the HS response in yeast (Rikhvanov *et al.* 2005) arguing against uncoupling of proton pumps as the primary mechanism for the chlorophenol-induced HS response.

In eukaryotes, the HS response has been suggested to result from cellular unfolding of labile proteins, which, upon recruiting Hsp70 and Hsp90, activates the HSF1 transcription factor (Morimoto 1998; Baniwal *et al.* 2004). Yet, chemical activation of the HS response under conditions where there is no significant protein misfolding (de Marco *et al.* 2005) argues against protein unfolding as the exclusive signal triggering the HS response. Indeed, when we tested *in vitro* the effects of high concentrations of TCP on the activity of purified LUC, no inhibition was observed (data not shown). This suggests that TCP is not proteo-toxic in itself, and is therefore unlikely to activate HSPs in the moss cells by way of unfolding labile proteins. DCP, TCP and PCP are increasingly hydrophobic. The fact that they could affect the photosynthetic efficiency as they did trigger the HS response suggests that these compounds can cross the plasma membrane and affect other inner hydrophobic compartments of the cell, such as the thylakoids. A possible mechanism for chlorophenol-mediated HS response could be by modifying the membrane state. Whereas artificial catalytic saturation of lipids in bacterial membranes can inhibit their HS response, the membrane fluidizer benzyl alcohol can elicit an HS-like response without temperature increase, by way of increasing the fluidity of the membranes as with HS (Horvath *et al.* 1998). Similarly, bimocmolol triggers the HS response by changing the membrane state (Torok *et al.* 2003). The hydrophobic partition coefficient ($\log K_{ow}$) of chlorophenols and their HS response-activating effects, increase with the number of chlorine atoms. This points at the lipophilicity of the chlorophenols as a possible factor in the HS inducing mechanism and in the long-term toxicity of these pollutants (Harvey *et al.* 2002; Czaplicka 2004).

In animal cells, a heat membrane receptor exists, named TRPV3. It is a calcium channel that can sense temperature-induced changes in the membrane state or that can be directly activated by chemical compounds such as capsaicin (Caterina *et al.* 1997; Xu *et al.* 2002). In *A. thaliana*, HS has been shown to cause a transient increase of cytosolic calcium (Gong *et al.* 1998), and inhibition of external calcium entry by ethyleneglycoltetraacetic acid (EGTA) has been shown to inhibit the HS response (Liu, Sun & Zhou 2005). It is therefore highly plausible that, like in animals, plants also possess heat-sensitive membrane receptors, which could be associated to calcium channels. The latter could detect changes in membrane state, either when naturally induced by temperature shifts or unnaturally induced by the action amphiphilic molecules, such as TCP or PCP. The notion of a membrane-bound receptor in plants could best explain the tight activation of the *hsp17.3B* promoter by temperature variations within the physiological range (22–28 °C) (Saidi *et al.* 2005) where protein unfolding is unlikely to occur. It could also explain the effect of small concentrations of molecules, such as PCP, showing strong synergism with mildly elevated temperatures. It should be noticed that the HS response can be induced by other specific chemicals from plant secondary metabolism, such as acetylsalicylic acid (Amici, Rossi & Santoro 1995) or celastrol (Westerheide *et al.* 2004), that may act downstream of the membrane in the HS signalling pathway (Table 1).

The notion of a membrane-associated HS sensor does not exclude that at high temperatures, or in the presence of chemicals interfering with protein synthesis or stability, the cellular protein unfolding state in the cell may also contribute and complement the HS response.

Mild heat treatment strongly enhances the sensitivity of HSP–GUS biosensor

When the chemical treatments were performed at moderately elevated, yet non-damaging temperatures, the ability of the HSP–GUS line to sense and react to low concentrations of toxic compounds was considerably increased. Interestingly, the minimal concentrations and durations of exposure sufficient to induce significant detectable effects were lower using the HSP–GUS biosensor than using fitness analyses (here, F_v/F_m ratio). In addition, this synergism identified some new, weak co-inducers of HS response such as AQSA, found to activate the stress promoter at 30 °C but not at 25 °C. This indicates that fixed toxicity thresholds cannot be set for specific pollutants, as they vary, for example, with temperature, and that the analytic measurements of pollutant levels in contaminated ecosystems or food do not suffice to assess the true biological consequences on organisms.

Although to assess the toxicity levels in samples from polluted ecosystems and industrial effluents, this plant-based bioassay can be used only under controlled laboratory conditions, it may significantly contribute to the

assessment of ecological risks and monitor the effective toxicity of pollutants in contaminated soils under simulated environments.

The introduction in the same HSP–GUS line of additional stress-inducible promoters, for example, from metallothionein and ascorbate peroxidase genes, using additional reporters such as green fluorescent protein and firefly LUC, may broaden the spectrum of this bioassay and increase its sensitivity with a multidimensional screen.

Interestingly, this moss bioassay reacts to a similar array of pollutants as well as pharmaceutical compounds than mammalian cells (Amici *et al.* 1995; Saidi *et al.* 2005). We observed that non-steroidal anti-inflammatory drugs that induce HSPs in human cells, also induce a similar HS-like response in plants (see sodium salicylate and celastrol, Table 1). The HSP–GUS line may thus contribute to the identification of new drugs that can specifically activate the chaperone network in eukaryotes to elicit the defence mechanism against toxic protein aggregates in ageing and many neurodegenerative diseases (Hinault *et al.* 2006).

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