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ORIGINAL ARTICLE

High frequency (900 MHz) low amplitude (5 V m^{-1}) electromagnetic field: a genuine environmental stimulus that affects transcription, translation, calcium and energy charge in tomato

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Abstract Using an especially-designed facility, the Mode Stirred Reverberation Chamber, we exposed tomato plants (*Lycopersicon esculentum* Mill. VFN8) to low level (900 MHz, 5 V m^{-1}) electromagnetic fields for a short period (10 min) and measured changes in abundance of three specific mRNA soon after exposure. Within minutes of electromagnetic stimulation, stress-related mRNA (calmodulin, calcium-dependent protein kinase and proteinase inhibitor) accumulated in a rapid, large and 3-phase manner typical of an environmental stress response. Accumulation of these transcripts into the polysomal RNA also took place (indicating that the encoded proteins were translated) but was delayed (indicating that newly-synthesized mRNA was not immediately recruited into polysomes). Transcript accumulation was maximal at normal Ca^{2+} levels and was depressed at higher Ca^{2+}, especially for those encoding calcium-binding proteins. Removal of Ca^{2+} (by addition of chelating agents or Ca^{2+} channel blocker) led to total suppression of mRNA accumulation. Finally, 30 min after the electromagnetic treatment, ATP concentration and adenylate energy charge were transiently decreased, while transcript accumulation was totally prevented by application of the uncoupling reagent, CCCP. These responses occur very soon after exposure, strongly suggesting that they are the direct consequence of application of radio-frequency fields and their similarities to wound responses strongly suggests that this radiation is perceived by plants as an injurious stimulus.

Keywords Mode stirred reverberation chamber · Radiofrequency electromagnetic field · Stress · Tomato · Wound-like responses

Abbreviations

AEC	Adenylate energy charge
BAPTA	Bis aminophenoxy ethane tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
EMF	Electromagnetic field
MSRC	Mode stirred reverberation chamber
RF	Radio frequency
RTqPCR	Real time quantitative PCR
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone

Introduction

The increased use of wireless communication devices over the last two decades has raised concerns that radio frequency electromagnetic fields (RF-EMF) may interact with living organisms and has led to a plethora of studies on the topic. Unfortunately, many of these studies have employed electromagnetic devices with inappropriate or uncontrolled

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emission parameters and a few demonstrate consistent effects of RF-EMF (Valberg et al. 2007). The majority of controversial RF-EMF studies have focused on complex responses such as human health or animal well-being. These depend on a multitude of different (and uncontrolled) factors and are concerned with long-term effects, thereby lessening the likelihood of a direct cause-effect relationship (Boice et al. 2002; Feychting et al. 2005). Plants are rarely used in RF-EMF experiments (Selga and Selga 1996; Tafforeau et al. 2002, 2004), partly because their very inertia makes the casual observer presume they are insensitive and thus unresponsive to their environment. In fact the opposite is more likely – their inability to escape the environment implies that plants must be able to sense subtle changes and adapt their responses accordingly (Vian et al. 1996; Ferguson 2004). Indeed, plants are able to sense various magnetic fields (Galland and Pazur 2005). Because they are non-motile, plants do not need to be constrained and because they are autotrophic, feeding/starvation does not impose an additional stress/variable. We have recently demonstrated that tomato plants react to RF-EMF and wounding in a similar manner and suggested that there may be a common mechanism behind these common responses (Roux et al. 2006; Vian et al. 2006; Beaubois et al. 2007). We have avoided the use of inappropriate or uncontrolled RF-EMF exposure by using the mode stirred reverberating chamber (MSRC; Roux et al. 2006; Vian et al. 2006): an especially constructed room, accepted as a standard for RF-EMF experiments, able to reproduce in a highly controlled and protected manner the characteristics of multi-directional RF-EMF urban radiation. Further, instead of measuring long-term, complex behavioural responses, we have chosen to measure rapid molecular responses (namely accumulation of stress-related transcripts and cellular energy state) that are more likely to be integral parts of the cause–effect relationship.

To accomplish this, tomato plants were exposed to short duration, low amplitude RF-EMF (900 MHz, 5 V m^{-1}, 10 min) and RTqPCR performed to assay the abundance of both total and polyribosome-associated transcripts, where assay of total mRNA measures net accumulation while assay of polysomal mRNA measures their current translation. Since all of the transcripts: calm-n6 (Depège et al. 1997), lecdpk1 (Chico et al. 2002) and pin2 (Pearce et al. 1991) are wound-up-regulated, we were able to further probe the similarities between wounding and RF-EMF treatment and since two of them (calm-n6, lecdpk1) encode calcium-binding proteins, we examined the effects of varying calcium availability to determine if there was any feedback mechanism involved in their transcription. Finally, we also measured ATP concentration and adenylate energy charge (AEC) after RF-EMF exposure, as well as the effect of a potent uncoupling agent, CCCP, on transcript accumulation, in order to assess the influence of RF-EMF on plant cell energy status and to correlate this with changes in mRNA abundances.

Materials and methods

Tomato plant culture

Seeds of *Lycopersicon esculentum* Mill. (cv VFN-8, obtained from INRA, Avignon, France and self-maintained in the laboratory greenhouse) were grown inside two identical custom-made (plywood) culture chambers with a hydroponic system and a light:dark photoperiod of 16:8 h, 26:21°C (175 µmol m^{-2} s^{-1} light intensity at the terminal leaf level provided by fluorescent tubes, Mazdafluor blanc industry Mazda-Philips, Paris, France) as previously described (Roux et al. 2006; Vian et al. 2006). Composition of the culture medium: macro-elements [85 µM NaCl; 0.55 mM $MgSO_4 \times 7\ H_2O$; 0.27 mM $(NH_4)_2SO_4$; 0.73 mM $Ca(NO_3) \times 4\ H_2O$; 1 mM KNO_3; 5.5 mM K_2HPO_4; 7.2 mM KH_2PO_4]; micro-elements [0.5 µM $(MoO_4(NH_4)_2$; 3.5 µM $MnSO_4\ H_2O$; 4.9 µM $ZnSO_4\ H_2O$; 9.1 µM H_3BO_3; 0.8 mM $CuSO_4\ H_2O$]; 2‰ iron chelate. Each of the chambers allowed to grow 48 plants (i.e. 12 independent experiments of four samples: C, 15, 30, 60.) In order to obtain not-exposed samples (related data are displayed as "shielded"), one of the culture chambers was armoured with a multi-layered aluminium material to protect the plants from exposure to RF-EMF (without influencing the conditions of culture, Beaubois et al. 2007).

RF-EMF treatment

When the fourth terminal leaf appeared (i.e. 3 weeks old), plants were RF-EMF-stimulated by placing the whole culture chamber (24 h prior the RF-EMF treatment) in the MSRC. The MSRC is a large (200 m^3) Faraday cage in which it is possible to emit, control and repeat the RF-EMF in a volume protected from external RF-EMF interferences (Roux et al. 2006; Vian et al. 2006; Beaubois et al. 2007). The aim of the MSRC is to create RF-EMF that illuminated plants from several directions with different polarizations (due to reflection properties of the MSRC). Plants are therefore placed in a volume where RF-EMF is homogeneous and isotropic (900 MHz; 5 V m^{-1}; 10 min). Control samples (C) were harvested immediately before starting the RF-EMF treatment and test samples (exposed and shielded) were harvested at different times after exposure (5, 15, 30, 60 min). The youngest terminal leaf of each plant was frozen in liquid nitrogen and constitutes a single sample (used either for RTqPCR or ATP measurement). Each result is expressed

as the mean of a minimum of three totally independent experiments ± the standard error.

Polysome and RNA isolation

Total RNA and polysomal RNA were extracted from the youngest terminal leaf. Polysomes were isolated by homogenizing tissue in 2× buffer U (400 mM Tris/HCl, pH 8.5; 400 mM saccharose; 60 mM $MgCl_2$; 120 mM KCl; 4 mM EGTA) containing 1% deoxycholate and 2.5% polyethylene-10-tridecylether, and pelleting the homogenate (with a TST-41.14 rotor, Kontron Ltd, Zurich, Switzerland) at 240.000*g* through a l ml pad of 50% saccharose in buffer B (40 mM Tris/HCl, pH 8.5; 20 mM KCl; 10 mM $MgCl_2$) according to described protocols (Davies and Abe 1995). Total RNA and polysomal RNA were purified with Tri-reagent (Sigma, http://www.sigmaaldrich.com) according to the furnished protocol.

RTqPCR analysis

RTqPCR (iCycler iQ—BioRad; with qPCR Mastermix Plus for SYBR Green I, Eurogentec, http://www.eurogentec.com) were performed after cDNA synthesis (Reverse Transcriptase Core Kit, Eurogentec) using total or polysomal mRNA as template. The abundance of targeted transcripts was normalised to the housekeeping actin mRNA and set relative to the control plant (C, not exposed, harvested just before the RF-EMF treatment) according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The accession numbers of targeted transcripts are: actin **BM956640**; calm-n6 **Y14764**; lecdpk1 **AF363784** and pin2 **AY129402**. To compensate plant-to-plant variability and to achieve statistical validity we performed triplicate real-time RTqPCR assays from at least three totally independent biological samples.

Calcium assay

Plants were transferred 24 h before the microwave stimulation onto fresh medium, either with regular calcium concentration (0.73 mM); calcium added (7.3 mM); calcium depleted (0 mM) or 0 mM calcium plus EGTA (0.5 mM), or BAPTA (0.4 mM) or $LaCl_3$ (0.1 mM).

Energy assay (ATP, AEC, CCCP)

ATP was extracted with perchloric acid (Keppler et al. 1970) and its concentration was measured with the ATP Bioluminescence Assay Kit CLS II (Roche, http://www.roche-applied-science.com) associated with a microplate luminometer (LB96V Berthold, http://www.berthold-ds.com). Adenylate energy charge (Atkinson 1968) was calculated after indirect enzymatic dosage of AMP and ADP (Pradet 1967) according to the following: AEC = (ATP + ½ ADP)/(ATP + ADP + AMP). When used, 200 μM of CCCP were added to a standard culture medium 90 min before RF-EMF experiment.

Statistical analysis

Statistical significance of the data was determined accordingly to the one-sided Mann–Whitney *U* test. Significant data (probability for the *U* value in the range 0.05–0.1) are indicated in the figure by an asterisk over the bars.

Results

Effects of RF-EMF on abundance of total and polysomal mRNA encoding stress-related genes

Figure 1 shows the relative accumulation of both total and polysomal mRNA of three stress-related markers: calmodulin-N6 (calm-n6; Fig. 1a), *Lycopersicon esculentum* calcium-dependent protein kinase (lecdpk1; Fig. 1b) and proteinase inhibitor II (pin2; Fig. 1c).

In the total RNA fraction (Fig. 1, treated), both calmodulin (Fig. 1a) and pin2 transcripts (Fig. 1c) exhibited the typical 3-phase stress response seen previously (Roux et al. 2006; Vian et al. 2006) with a maximum increase of 4.0- to 5.5-fold within 15 min, a decline at 30 min followed by a second increase at 60 min to 3.5- to 6.5-fold. The lecdpk transcript, however, exhibited a delayed 2-phase response, reaching a maximum of fivefold at 30 min before declining at 60 min (Fig. 1b). Changes in polysomal mRNA (Fig. 1, treated) were different from total RNA, but essentially identical to each other. In all cases, there was a slow increase, reaching a maximum of fourfold to sixfold the original level at 60 min. This indicates that there is a delay of at least 30 min between maximal accumulation of mRNA and its recruitment into polysomes. No significant change in total or polysomal mRNA accumulation was ever seen in shielded plants, i.e. plants treated in the aluminium armoured culture chamber (Fig. 1, shielded).

Requirement for optimal calcium for RF-EMF-evoked gene expression

Recent work from our laboratory (Beaubois et al. 2007) using a calcium channel blocker and a calcium chelating agent, has shown that calcium is needed for RF-EMF-evoked expression of pin2 and lebzip1 mRNA. We wondered if this was true for other RF-EMF-induced stress genes and if so, how crucial was the calcium availability. Accordingly, we assayed the same three stress-related

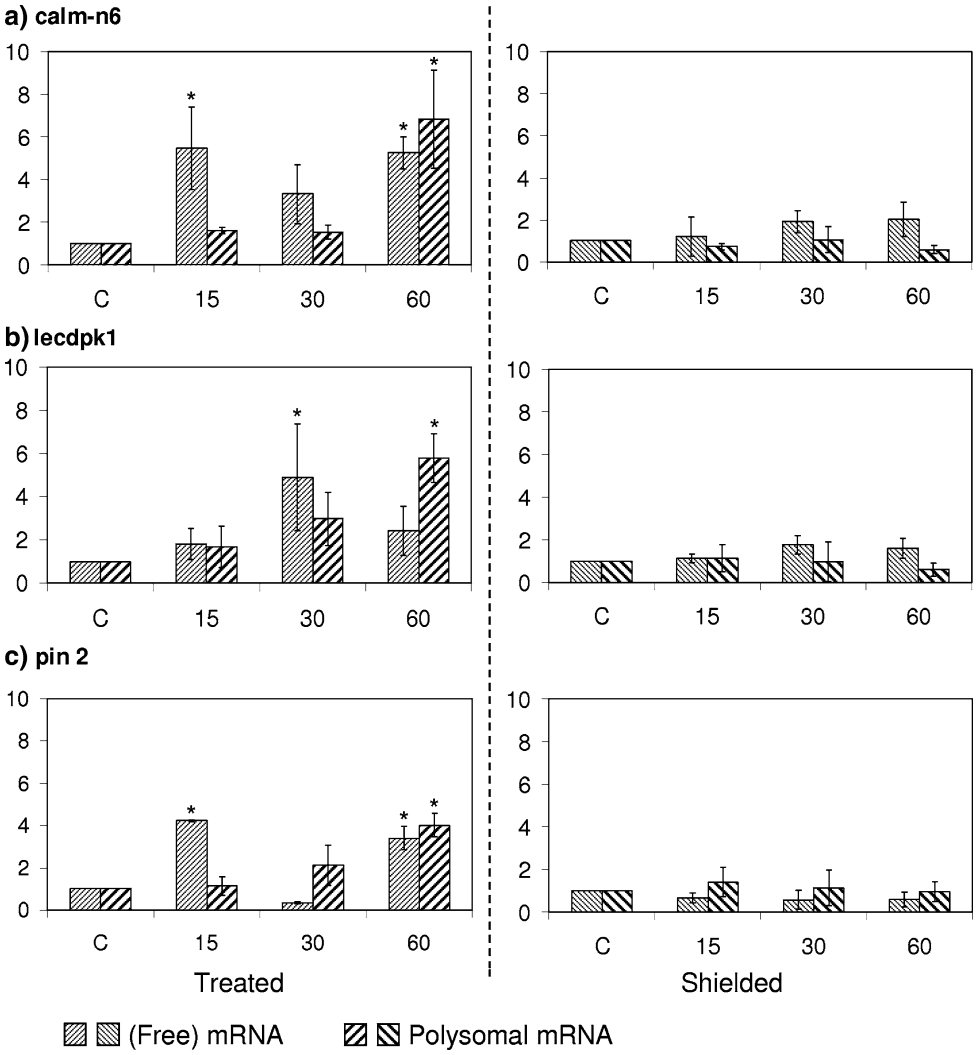

Fig. 1 Relative changes in total mRNA and polysomal RNA abundance following the RF-EMF exposure. Plants were grown in the standard culture chamber (*treated*) and in the aluminium armoured culture chamber (*shielded*). Targeted transcripts (**a** calm-n6; **b** lecdpk1; **c** pin2) where *bars* represent mean values ± SE from at least three independent experiments

transcripts used above (Fig. 1) under several conditions of calcium availability looking specifically at the early response—5 and 15 min after exposure. For all the following experiments, the shielded plants (grown in the armoured culture chamber) did not accumulate transcripts to levels significantly higher than the control (data not shown).

When plants were grown in culture medium containing regular amounts (0.73 mM) of calcium, the levels of pin2 and lecdpk1 transcripts remained essentially unchanged 5 min after termination of the treatment, while the calm-n6 transcript increased more than threefold (Fig. 2a). However, 15 min after exposure, the level of pin2 transcript had increased more than threefold, while both calcium related transcripts (lecdpk1 and calm-n6) had increased fivefold (Fig. 2a). When plants were grown in liquid medium containing 7.3 mM calcium (ten times the normal concentration), accumulation of pin2 (Fig. 2b, dark grey bar) was reduced compared with the regular calcium level (Fig. 2a, dark grey bar), but still showed a slight elevation (near twofold) at 5 min and a significant (almost threefold) increase at 15 min. However, accumulations of both the calcium-related transcripts (Fig. 2b, white and light grey bars) were totally suppressed.

When plants were grown with reduced amount of calcium, no significant decrease was found for any transcript (Fig. 2c). When calcium uptake was prevented either by adding the ion-chelating agent, EGTA (Fig. 2d), the very specific calcium-chelating agent, BAPTA (Fig. 2e) or the calcium channel blocker lanthanum chloride, $LaCl_3$ (Fig. 2f), no transcript accumulation occurred and in the case of pin2 in EGTA 5 min after exposure, the level was severely reduced compared with the control (Fig. 2d).

Effects of RF-EMF on the energy status

Since both RNA synthesis (as estimated from accumulation of total RNA) and protein synthesis (whose potential is estimated from polysomal mRNA) are energy-consuming reactions, we decided to measure the ATP concentration and

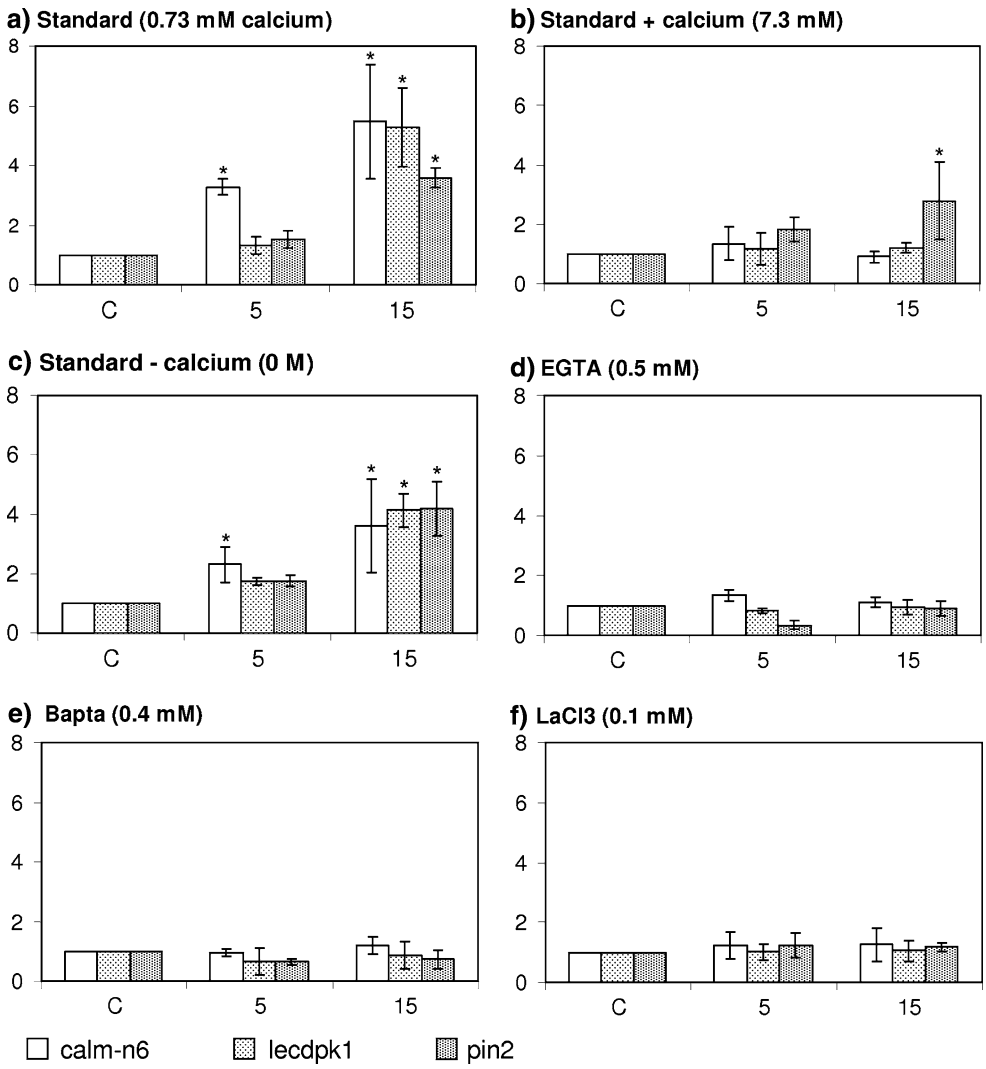

Fig. 2 Effect of varying the availability of calcium on early transcript accumulation in response to the RF-EMF exposure. **a** Plants maintained on standard medium (0.73 mM of calcium); or **b** transferred to medium with tenfold extra calcium (7.3 mM). **c** Devoid of calcium (0 mM). **d** Devoid of calcium (0 mM) and containing 0.5 mM of EGTA (cathion—Ca^{2+} preferred—chelating agent). **e** Devoid of calcium (0 mM) and containing 0.4 mM of BAPTA (a calcium chelating agent). **f** Devoid of calcium (0 mM) and containing 0.1 mM $LaCl_3$ (a calcium channel blocker). Bars represent mean values ± SE of RF-EMF exposed samples from at least three independent experiments

AEC after RF-EMF exposure (Fig. 3). The ATP concentration was unchanged for the first 15 min after exposure, but then declined significantly by about 40% after 30 min and remained close to that level at 60 min (Fig. 3a). The AEC was also unchanged for the first 15 min and declined significantly to a ratio of 0.63 at 60 min (Fig. 3b). The AEC values for the shielded plants were all within the ratio of 0.8–0.9 which indicates that our culture conditions (within the enclosed culture chambers) were well suited to tomato plants.

The addition of the protonophore CCCP (200 μM), a highly effective inhibitor of ATP synthesis (Heytler 1963), to the culture medium 90 min before RF-EMF exposure did not trigger by itself any stress response (Fig. 4). Indeed, in the presence of CCCP the plants protected from RF-EMF (in the armoured chamber) did not show any mRNA accumulation. However, this uncoupler led to a total inhibition of the transcript accumulation previously observed (Fig. 1) since plants exposed to RF-EMF displayed mRNA abundances indistinguishable from the shielded plants (Fig. 4).

Discussion

Tomato plants usually respond to wounding by accumulating stress-related transcripts in a typical 3-phase response: phase 1, initial period of rapid accumulation; phase 2, subsequent period of transcript decline; phase 3, second period of accumulation (Davies et al. 1997; Vian et al. 1999; Stankovic et al. 2000). During accumulation (phase 1, 3), transcription must exceed RNA degradation, while during phase 2 (transcript decline), RNA degradation must exceed RNA synthesis. This most likely results from enhanced RNase activity, which is known to be increased after injurious treatments (LeBrasseur et al. 2002). In the present work, although they are a downstream event in the signal transduction pathway, all three transcripts exhibited the typical 3-phase response with similar kinetics. Recruitment of mRNA into polysomes (translation) is not necessarily an immediate consequence of its synthesis (transcription; Davies and Larkins 1980). The data here exemplify this (Fig. 1). The period of maximum accumulation of the

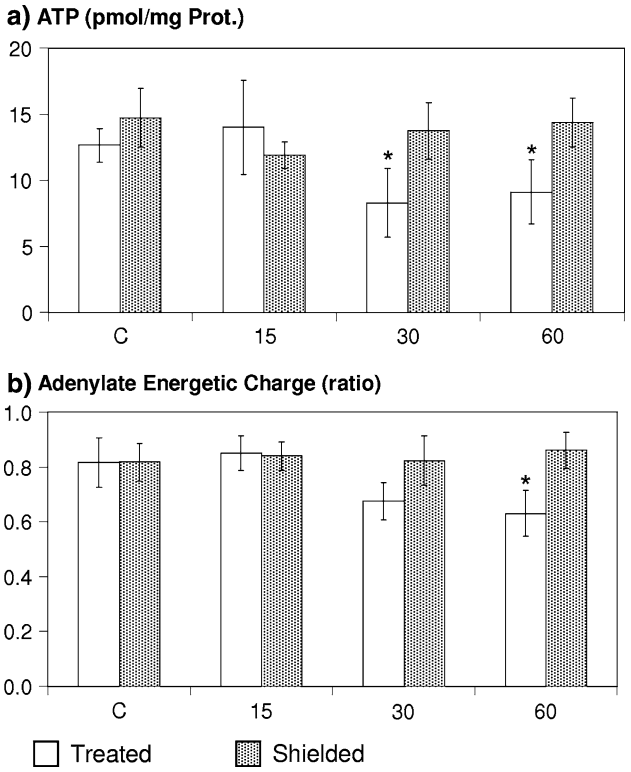

Fig. 3 Changes in ATP concentration and adenylate energy charge after RF-EMF exposure. Plants were grown in the standard culture chamber (*treated*) and in the aluminium armoured culture chamber (*shielded*). **a** ATP concentration (pmol/mg Prot.). **b** Adenylate energy charge (ratio). Bars represent mean values ± SE from at least three independent experiments

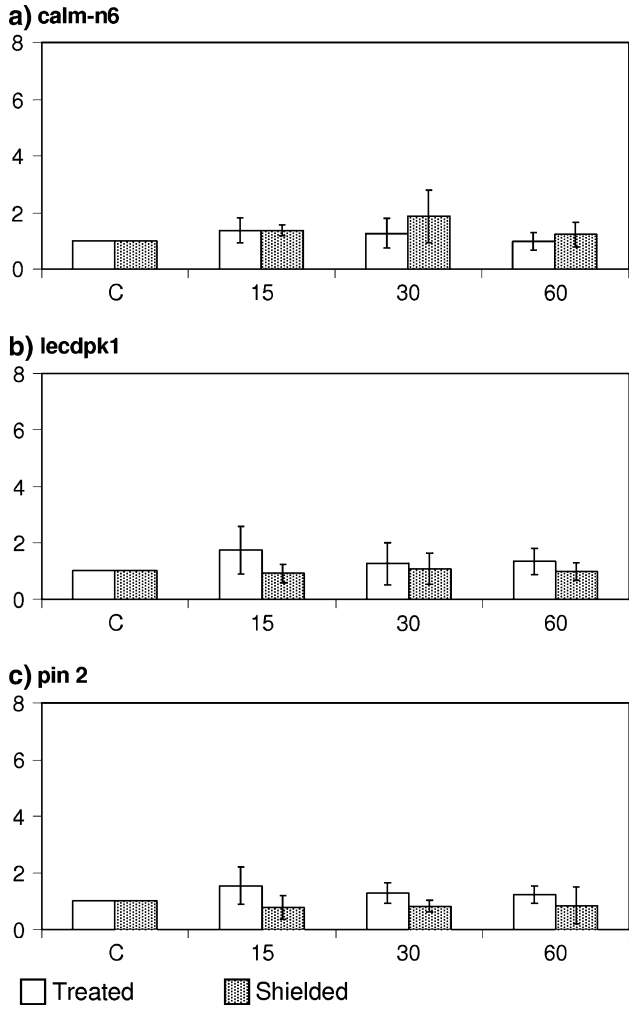

Fig. 4 Effect of the uncoupler CCCP (200 μM) on transcript abundance in response to RF-EMF exposure. Plants were grown in the standard culture chamber (*treated*) and in the aluminium armoured culture chamber (*shielded*). Targeted transcripts (**a** calm-n6; **b** lecdpk1; **c** pin2) where bars represent mean values ± SE from at least three independent experiments

stress-related transcripts in the total RNA fraction, which occurs by 15 or 30 min precedes that of accumulation into the polysomal RNA fraction (recruitment into polysomes) by at least 30 min. Similar enhancement of mRNA recruitment into polysomes has been described previously after a mild wound treatment (cotyledon pricking; Henry-Vian et al. 1995). These results do support the suggestion that the newly transcribed RNA is indeed translated and that the encoded proteins may well play a role in defence of the plant.

Here we focused on the effects of elevated calcium levels and on lowered available calcium using several different calcium-counteracting drugs applied individually to the culture medium of whole plants subjected to RF-EMF treatment (Fig. 2). When direct comparisons are made between responses to various calcium levels, clear patterns emerge. The transcripts encoding calcium-binding proteins (calm-n6 and lecdpk1) are maximally accumulated only at regular calcium levels, whereas the non-calcium-related transcript, pin2, is essentially unaffected at greatly elevated or slightly reduced calcium levels. In high calcium condition (7.3 mM), calcium-related protein transcripts (calmodulin and CDPK) do not accumulate after RF-EMF exposure, yet Pin-2 transcripts do, suggesting that the latest is less affected by high calcium. When all calcium was removed from the medium and available calcium lowered even more by adding the chelating agent EGTA, a highly specific calcium-chelating agent BAPTA, or a calcium channel blocker $LaCl_3$, there was no increase in any transcript. These drugs, which typically influence calcium-related genes expression (Leitner-Dagan and Weiss 1999), also inhibit transcript accumulation in response to wounding (Vian et al. 1997) and RF-EMF exposure (Beaubois et al. 2007). These observations support previous findings that calcium is essential for gene expression via its association with many transcription regulators and its requirement for RNA polymerase II activity (Coulon and Blanchard 2001). Moreover, these results also agree with the important but not exclusive role of external calcium in cell stress signalling and regulation

of cytoplasmic calcium concentration (Knight et al. 1997). Several hypotheses have been proffered concerning how RF-EMF could lead to Ca^{2+} modulation: by direct activation of the signal perception pathway (Trewavas 2000), by acting on the membrane-cytosol interface and/or by acting directly on the ion itself (Goodman and Blank 2002) or on ion channels (Aldinucci et al. 2003).

It is apparent that the energy state of the plants was affected by the RF-EMF treatment since it caused a decrease in ATP concentration and AEC (0.63) after 30 min (Fig. 3). AEC is a very sensitive parameter reflecting the energy state of cells. An organism maintained in suitable conditions usually displays AEC values in the range of 0.8–0.9. Any variation from this range is a consequence of modifying energy metabolism (Moal et al. 1991). The shielded plants here were all within these values (±0.84), however the RF-EMF-exposed plants displayed at least 30% decrease. Similar transient energy variations have been observed after leaf-wounding experiments, especially a 30% decrease of AEC (Henry-Vian et al. 1996). It has been shown that ATP is one of the major mediators of stress transduction within the plant and that it is able to regulate the level of stress-related mRNA (Jeter et al. 2004). The decrease in ATP can be explained since it is immediately mobilised by many cellular process (especially RNA and protein synthesis) as soon as a stress event occurs (Dobrota 2006) and decreases in AEC occur after cell stress, especially anoxia (Blokhina et al. 2003). However, the adenylate nucleotide pool (ATP + ADP + AMP) remained at a steady-state level during our experiments (data not shown) suggesting that this energy decrease did not affect cell metabolism in a profound manner. It is highly likely the partial depletion of ATP results from its consumption during the energy-dependent processes of transcription and translation. By breaking the proton gradient with the use of the protonophore CCCP, with its subsequent lowering of ATP synthesis, the typical RF-EMF-evoked mRNA accumulation was completely suppressed (Fig. 4) implying a direct link between the energy potential of the cell and the genesis of the stress responses.

Injurious or non-injurious stimulations evoke an almost immediate modification of membrane potential that is transmitted through the plant as an action potential or a variation potential, depending upon the strength of the stimulus. These electrical signals have been formally linked to the accumulation of the stress-related transcript, Pin (Wildon et al. 1992; Stankovic and Davies 1997; Herde et al. 1999), calmodulin (Vian et al. 1996) and CMBP (Vian and Davies 2006). Calcium counteracting drugs reduced the amplitude of these electrical signals (Julien et al. 1991) and suppressed the accumulation of calmodulin transcripts after wounding (Vian et al. 1997). It has also been shown that CCCP causes a strong membrane depolarization (Lew and Spanswick 1984). When plants are exposed only locally (i.e. on the oldest leaf), the rest of the plant (protected from RF-EMF inside the armoured culture chamber) also shows almost identical stress-related transcript accumulation, suggesting the very rapid transmission of an informative signal (Beaubois et al. 2007). The genesis and/or transmission of this signal is exceedingly rapid and strongly dependent upon calcium. These characteristics suggest that an electrical wave of depolarisation is produced after exposing plants to RF-EMF (Beaubois et al. 2007). Taken together, these results imply the involvement of membrane potential in both the local and distant responses of tomato to RF-EMF exposure. This is consistent with many reports, which propose that the plasma membrane is the primary site of interaction between living organisms and RF-EMF (Galvanovskis and Sandblom 1997; Astumian 2003). This could also imply that energy is needed to generate the systemic electrical signal thought to be the mediator of the whole plant responses to wounding (Davies and Stankovic 2006) and RF-EMF exposure (Beaubois et al. 2007), again supporting the injury-like effect (Tafforeau et al. 2004; Roux et al. 2006; Vian et al. 2006) of microwaves on plants.

Taken as a whole, the data provide new evidence supporting the hypothesis that plants perceive and respond to microwave irradiation as though it was an injurious treatment. Even though the RF-EMF is non-thermal and the total power we used very low (0.1 W dissipated in 200 m^3), the similarities with wounding (leaf-pricking, burning and cutting) are striking. In addition to the rapidity of the response and its dependency on the second messenger calcium, we observed a strong correlation between all the parameters measured (total and polysomal transcript abundance, ATP concentration and AEC). This suggests a functional relationship between them all. Further work will help in understanding the transduction pathways involved and how the RF-field interacts with the cell.

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