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Electromagnetic fields (900 MHz) evoke consistent molecular responses in tomato plants

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Although the effects of high-frequency electromagnetic fields on biological systems have been studied frequently, unequivocal results have rarely been obtained, primarily because suitably controlled experiments could not be performed. In the present work, tomato plants were exposed to a homogeneous and isotropic field (900 MHz) using a mode stirred reverberation chamber, and the stress-related transcripts (calmodulin, protease inhibitor and chloroplast mRNA-binding protein) were assayed by real-time quantitative PCR. Exposure to an electromagnetic field induced a biphasic response, in which the levels of all three transcripts increased four- to six-fold 15 min after the end of electromagnetic stimulation, dropped to close to initial levels by 30 min, and then increased again at 60 min. We deliberately focused on the very early molecular responses to high-frequency electromagnetic fields in order to minimize secondary effects.

Introduction

The development and increased use of wireless communication technologies in recent years have aroused suspicion that there may be hazardous effects of High-Frequency (HF) Electromagnetic Fields (HF-EMFs) on living systems, including humans (Elwood 2003). A wide range of EM signals emitted by the global system for mobile phone (GSM) networks are mixed in urban areas locally displaying an HF-EM environment with amplitudes of several volts per meter. Frequently, mobile phones or mobile phone-like devices are used for eletromagnetic exposure experiments (Dasdag et al. 2003, Haarala et al. 2003, Irmak et al. 2002, Weisbrot et al. 2003), as are other emitting devices, such as transversal electromagnetic cells (Marinelli et al. 2004), parallel plate resonators (Mashevich et al. 2003), wave guides (Czyz et al. 2004) and anechoic chambers (Gos et al. 2000). We utilized a Mode Stirred Reverberation Chamber (MSRC), since it has recently been recognized in the IEC 61000-4-21 standard (IEC 2003) as a stimulation device. The main advantages of the MSRC are that it provides protection from environmental EMF and creates a statistically isotropic and homogeneous EMF similar to that found in urban environments (Otterskog and Madsén 2003), ensuring that experiments are directly related to the sole variable, that of EMF exposure.

The majority of EMF studies, especially in epidemiology, have led to highly conflicting conclusions (Elwood 2003; Levin 2003). For example, human behaviors such as cognitive performance (Haarala et al. 2003) and comportment (D'Andrea et al. 2003) did not yield conclusive results, because of the difficulty in evaluating psychology-related parameters. Similar kinds of problem have been encountered with animals (mainly rabbit and rat by Irmak et al. 2002 and Dasdag et al. 2003), due to the stress generated by the experimental protocol. Data obtained from culture of immunity-related cells (Aldinucci et al. 2003; Marinelli et al. 2004; Mashevich

Abbreviations – AP, Action Potential; EMF, Electromagnetic Field; GSM, Global System for Mobile phone; HF, High Frequency; MSRC, Mode Stirred Reverberation Chamber; RT-qPCR, Real-Time quantitative PCR; SEM, Standard Error of the Mean; VP, Variation Potential.

et al. 2003) are the most convincing: they show effects of HF-EMF at the cellular or molecular level (such as decrease in DNA stability, Ca^{2+} movement, deregulation of apoptosis). However, an intact organism is preferable to cultured cells, since the former maintains its full potential for signal perception, transduction and response. With this in mind, we focused our attention on the tomato plant (*Lycopersicon esculentum*), the model system for studying plant responses to environmental stresses such as wounding, in order to circumvent any psychological factors and to allow control of all potential environmental variables (i.e. light, temperature, nutrients, handling) that may modulate perception of, or response to, the EMF stimulus. Plants, notably flax, have already given noteworthy results in response to HF-EMF (Tafforeau et al. 2002, 2004, 2006).

Many studies focused on animal behavior and cancer have furnished contradictory results because of the difficulty in identifying relevant parameters that are measurable in a rapid and repeatable manner. This contrasts with experiments associated with clearly defined parameters such as oxidative stress (Irmak et al. 2002), gene expression (Czyz et al. 2004), Ca^{2+} movement (Aldinucci et al. 2003), genomic stability (Marinelli et al. 2004), or heat shock proteins (Weisbrot et al. 2003), which generate reproducible data. However, many of these studies measured responses after hours (Marinelli et al. 2004), days (Mashevich et al. 2003) or even months (Dasdag et al. 2003) following the electromagnetic stimulus. In striking contrast, we were interested in the very rapid molecular responses following electromagnetic stimulation, in order to minimize side effects and the possible influence of other factors. To do this, we measured changes in the abundance of three previously identified wound-induced transcripts that are known to play a role in the early events of plant responses to stress. Here we show that a non-thermal HF-EMF (900 MHz, 5 V m^{-1}) is able to evoke rapid accumulation of these transcripts. Future work will be directed towards the global analysis of microwave-induced gene expression (microarrays) and comparison with other stresses.

Materials and methods

Plant culture

Tomato plants (*Lycopersicon esculentum* cv. VFN-8) were grown inside a plywood custom-made culture chamber for 3 weeks (until the fourth terminal leaf appears). A controlled hydroponic system was used for culture with a light/dark photoperiod of 16 : 8 h, 26 : 21°C (150 μmol m^{-2} s^{-1} light intensity, Mazdafluor blanc industrie, Mazda-Philips, Paris, France).

Electromagnetic stimulation

The MSRC is a system with several essential components constructed especially to give reproducible EMF stimulation (Fig. 1). It consists of: a large room with a double-layered steel wall, acting as a Faraday cage and protecting experiments from environmental electromagnetic pollution; an antenna to generate the EMF; movable blades to stir the EMF so that it is statistically homogeneous and isotropic in a determined working volume; and a control panel to generate the appropriate EMF with the following characteristics: frequency, 900 MHz non-modulated; amplitude, 5 V m^{-1}(average signal amplitude of a GSM telephone) and 40 V m^{-1} (close to the French legal emission limit); duration, 2–10 min (within the duration of a GSM phone call). The culture chambers containing plants were placed in the working volume of the MSRC 24 h prior to EMF exposure, to avoid possible stress responses due to the moving procedure. The wooden culture chamber had no major influence on the EMF characteristics (homogeneity and isotropy) received. In control (i.e. 'shielding') experiments, plants were placed in the culture chamber enclosed in a polymer mesh covered with aluminum foil to prevent exposure to EMF (Vian et al., 2006). In all cases, the fourth leaf of control and treated plants was harvested at the appropriate time and immediately frozen in liquid nitrogen. A single plant was used for each point of the kinetic, and the experiments were independently repeated at least three times. The plants were discarded after tissue collection.

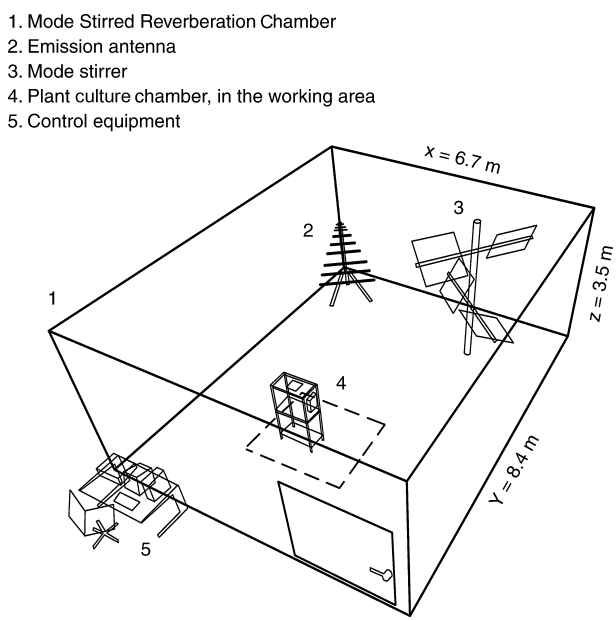

Fig. 1. The MSRC equipment especially designed to generate homogeneous and isotropic HF-EMF.

mRNA extraction and cDNA synthesis

mRNA extraction was performed using Tri-reagent (Sigma), and the 'advantage RT-for-PCR kit' (BD Biosciences) was used for cDNA synthesis, both according to the manufacturer's instructions.

Real-time quantitative PCR (RT-qPCR) analysis

Amplifications were conducted on an iCycler iQ (Bio-Rad) with the qPCR Mastermix Plus for SYBR Green I (Eurogentec). The abundance of targeted genes transcripts was normalized to *actin* mRNA and set relative to the control plant (C, not exposed, harvested before electromagnetic exposure) according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The accession numbers of targeted genes are: *actin*, BM956640; *calmodulin-N6*, Y14764; chloroplast mRNA-binding protein (*cmbp*), AF106660; and proteinase inhibitor (*pin2*), AY129402.

Results

Exposure of tomato plants to an EMF of 900 MHz, 5 V m^{-1} for 10 min led to changes in abundance of all three stress-related transcripts, *calmodulin-N6*, *cmbp* and *pin2* (Fig. 2, black bars). *Calmodulin-N6* (Fig. 2A) and *cmbp* mRNA (Fig. 2B) accumulated strongly (5.5- and 6.6-fold respectively) by 15 min, declined to levels indistinguishable from those in the shielded plants at 30 min, and showed somewhat weaker accumulation at 60 min (5.3- and 5.1-fold respectively). The *pin2* transcript gave slightly smaller responses, 4.2-fold at 15 min and 3.4-fold at 60 min (Fig. 2C). When plants were totally shielded from the EMF in the aluminum-enclosed culture chamber, the accumulation of mRNA was strongly reduced (Fig. 2. white bars), verifying that the responses did, indeed, result from EMF exposure.

In order to determine if there was a dose–response relationship between the EMF applied and the amount of transcript accumulated, we subjected plants to the dose used in Fig. 2 (5 V m^{-1} for 10 min), a dose of higher amplitude (40 V m^{-1} for 10 min), a dose of shorter duration (5 V m^{-1} for 2 min), and no dose (shielded plants), and measured *pin2* mRNA accumulation (Fig. 3). Furthermore, to determine how rapidly transcript accumulation occurs, plants were also harvested immediately (0 min) and 5 min after EMF exposure. Transcript accumulation was essentially identical at the two higher doses (Fig. 3, black bars and gray bars), being about five-fold at 15 min and four-fold at 60 min, but there was no transcript accumulation at the low dose (Fig. 3, hatched bars) or in the shielded plants (Fig. 3, white bars). No transcript accumulation occurred prior to 15 min (Fig. 3).

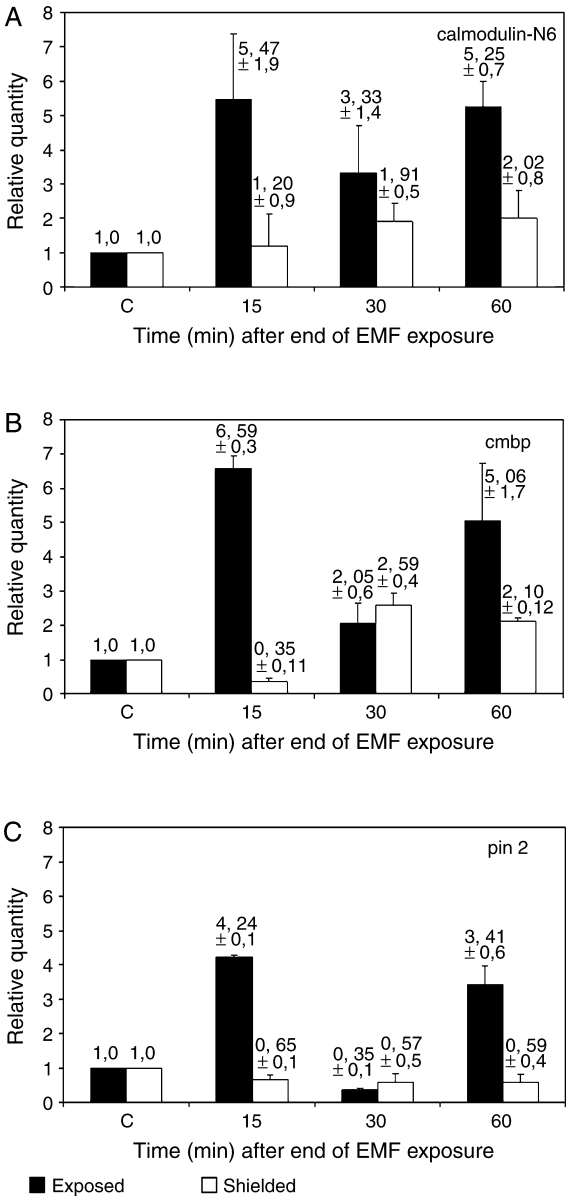

Fig. 2. Abundance of stress-related transcripts after exposure to EMF. Tomato plants were grown as described in Materials and methods and transferred into the MSRC 24 h prior experiment: EMF exposure for 10 min at 900 MHz (black bars), or shielding from exposure (white bars). At different times after exposure, RNA was isolated; the amount of transcript encoding *calmodulin-N6* (A), *cmbp* (B) and *pin2* (C) was quantified by RT-qPCR and normalized to the amount of the housekeeping transcript, *actin*. Values are expressed relative to the control (not exposed plant) value. Bars represent mean values ± SEM from at least three independent experiments.

Discussion

The purpose of this study was to determine whether short exposure (10 min) of a plant to low-level (5 V m^{-1}) HF-EMF (900 MHz), similar to that used in cell phones, could evoke a rapid biological response at the molecular level

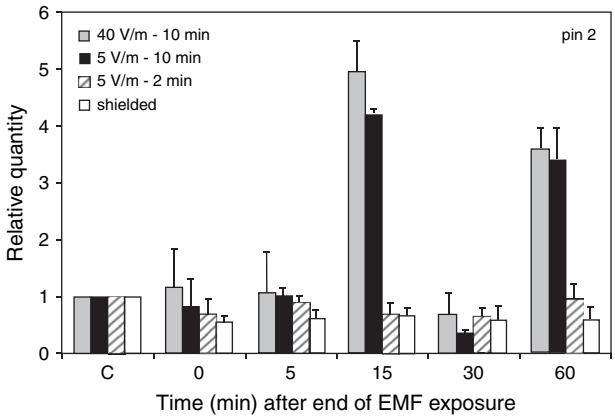

Fig. 3. Abundance of *pin2* transcripts after exposure to different amplitude/duration of EMF. Conditions are the same as Fig. 2. *pin2* transcript were measured and EMF was applied at 40 V m^{-1} for 10 min (black bars); 5 V m^{-1} for 10 min (gray bars), or 5 V m^{-1} for 2 min (hatched bars), or the plants were not exposed (shielded) (white bars).

in tomato plants. We think that EMF could constitute a genuine environmental stimulus/stress for tomato plants, insofar as it evokes rapid and strong molecular responses – the accumulation of stress-related transcripts. Our results tend to show a direct relationship between HF-EMF exposure of tomato plants and responses at the level of gene expression. Although not identical, the kinetics and amplitudes (three- to seven-fold increases) of the targeted transcripts showed striking similarities with the previously described physiologic responses following injurious treatments such as leaf flaming or electrical stimulation (Stankovic and Davies 1997, Vian et al. 1999). Accordingly, we propose here that HF-EMF exposure may constitute an environmental stimulus for the tomato plants.

Calmodulin is the major cell Ca^{2+} receptor and plays a central role in the early events of cell stress responses (Yang and Poovaiah 2003), while *cmbp* and *pin2* are expressed after wounding (flaming, insect bite) (Vian et al. 1999, Zhang et al. 2004). The fact that they all accumulate rapidly indicates that plant stress response pathways are affected by HF-EMF. Surprisingly, all transcripts showed the maximum accumulation at the same time point (15 min after the end of the electromagnetic exposure), and had the same accumulation profile (two distinct peaks separated by a sharp decrease). These similarities (in terms of kinetics and amplitude) indicate the existence of a primary signal able to trigger the molecular response. Such biphasic accumulation of stress transcripts has been observed before in response to flame wounding (Peña-Cortès et al. 1995) and has led to various hypotheses concerning the nature of the primary signal: chemical, hydraulic or electrical, or a combination of these (Davies and Stankovic, 2006). Indeed, it is quite possible that three distinct signals are involved: the first stimulating the initial synthesis of the transcripts; the second evoking their degradation; and the third stimulating the later burst of synthesis.

The interaction between EMF and the biological sample is not yet understood (Lacy-Hulbert 1998). Frequently, it appears that most of the observed biological responses are due to a 'thermal' effect (D'Andrea et al. 2003, Meltz 2003). The energy used here in the electromagnetic exposure was very low (close to 0.1 W dissipated in 200 m^3) and did not produce any thermal effect. It is important to note that duration (10 min), amplitude (5 V m^{-1}), homogeneity and isotropy of the EMF exposure were representative of a standard mobile phone emission.

The shielding experiments showed reduced accumulation of the tested transcripts (Fig. 2). This result could also indicate a slower plant response to the remaining EMF (0.5 V m^{-1}) present in the aluminum enclosed culture chamber. This interpretation may explain the consistent accumulation of *cmbp* transcripts (particularly at 30 min) in the shielded chamber.

The amplitude of 40 V m^{-1} is close to the maximum authorized emission of GSM base antennae according to International Commission on Non-Ionizing Radiation Protection guidelines (ICNIRP 1998). Surprisingly, no significant differences occurred in the kinetics or levels of *pin2* mRNA accumulation between 5 V m^{-1} and 40 V m^{-1} exposure (Fig. 3). Interestingly, decreasing the duration of exposure to EMF (from 10 to 2 min), at a fixed amplitude of 5 V m^{-1}, suppressed the molecular response (Fig. 3). There is therefore no direct link between the amplitude of the stimulation and the amplitude of the plant response. These results suggest the concept of an 'all-or-nothing' response, which is characteristic of the action potential (AP), the only 'genuine' electrical signal found in plants (Bowles 1995, Davies 2006). However, the propagation of a variation potential (VP), produced after an injurious stimulation, could also be implicated. Specific investigations using electrophysiological methods would be required to determine this. Preliminary results indicate that a rapid signal is actually transmitted through the plant after local stimulation (data not shown).

Our results concerning *calmodulin-N6* suggest that variations in cytoplasmic and membrane-neighboring Ca^{2+} concentrations are early events in plant responses to EMF stimuli. The EMF could interact with moving charges or charged species (Levin 2003) such as hydrogen ions, which are implicated in various biological processes. Moreover, HF-EMF may lead to ion movement, directly or indirectly, and particularly near the plasma membrane (Lacy-Hulbert et al. 1998), and this could initiate the biological response. This 'ionic' explanation is particularly attractive when considering the ionic (Ca^{2+}, Cl^-, K^+) nature of the plant AP (Davies 2006, Davies and Stankovic 2006).

This work suggests the existence of a formal connection between HF-EMF exposure of intact plants and very rapid molecular responses. Assuming that a stress response is really demonstrated, actual damage could occur, but this has not yet been proven (the HF-EMF does not cause any obvious physical tissue damage). The basic claim that low-intensity HF-EMF actually causes a measurable reaction in plant should not be underestimated, or overestimated. In particular, the relevance of these observations to other biological systems must not be overstated. Finally, the ultimate goal of this work is to use microarrays to identify the similarities and differences between microwave-induced genes and those induced by injurious treatments such as flame wounding (Stankovic and Davies 1997, Vian et al. 1999).

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