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Role of a Ca^{2+}-ATPase induced by ABA and IAA in the generation of specific Ca^{2+} signals

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Abstract

The control of the Ca^{2+}-ATPase gene (*LCA1*) that encodes two different membrane-located isoforms by two antagonic phytohormones, ABA and IAA, has been investigated. Strikingly both the growth regulators induce the *LCA1* expression. By using a protoplast transient system, the *cis*-acting DNA elements responding to both, abiotic stress (ABA) and normal development (IAA), are dissected. ABA triggered a 4-fold increase in the GUS-activity. A single ACGT motif responsible for most of the *LCA1* mRNA induction was localized at an unexpectedly large distance (1577 bp) upstream of the translational start. In the case of IAA, although there is a TGTCTC sequence that is known to be an important *cis*-acting element, two TGA motifs play a more critical role. It is proposed that the Ca^{2+}-ATPase isoforms might intervene in the generation of specific Ca^{2+} signals by restoring steady-state Ca^{2+} levels, modulating both frequency and amplitude of Ca^{2+} waves via wave interference.

Keywords: Calcium; ABA; IAA; Ca^{2+}-ATPase; ABRE; *LCA1*; *Lycopersicum esculentum*; Calmodulin binding domain

The concentration of cytosolic free calcium is critically important for the control of many essential cellular responses, therefore it has to be strictly regulated to gain an accurate function of the cell. Such a specific regulation of many different events has to be carried out by not only modulating the concentration, but also in a spatial and temporal mode, in the manner of Ca^{2+}-waves [1]. Consequently, compartmentalization plays a significant role in Ca^{2+}-related signal transduction events. Measurements of cellular Ca^{2+} have shown that plant cells avoid potentially toxic effects of Ca^{2+} by maintaining levels in both the cytosol and nucleus, that are 3–4 orders of magnitude lower than the levels in other cellular compartments. According to the intracellular messenger theory, Ca^{2+} along with a small group of other compounds plays a fundamental role in signal transduction by communicating signal perception at a localized receptor to other parts of the cell, where the effectors of the cellular response are located. The increased Ca^{2+} level promotes the formation of Ca^{2+} complexes with target proteins (such as calmodulin) that, in turn, regulate the activity of the effector proteins which determine cell response. An increase in cytosolic free Ca^{2+} has been observed in several cell types in response to a number of stimuli. Some examples of which are: plant hormones, gravity perception, mechanical stimuli, high and low temperatures, electrical fields, apoptosis, exposure to oxidative agents, red light, fungal elicitors, self-incompatibility factors, salt stress, etc [2]. In plants of *Arabidopsis thaliana*, active Ca^{2+} transport from (and inside) the cytosol is catalyzed by different transport pathways. Transport is mainly carried out by P-type ATPases found on the plasma membrane (PM), tonoplast (TN), endoplasmic reticulum (ER), plastid membranes, and perhaps on the Golgi apparatus. They are classified into two types: ER-type calcium ATPases

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and autoinhibited calcium ATPases. The Ca^{2+}-permeable channels (localized at the PM) also participate and are constituted by: non-selective cation channels, depolarization activated channels, and hyperpolarization activated channels (characterized at an electrophysiological level rather than a molecular level), a two-pore channel, cyclic nucleotide-gated channels (CNGC), and glutamate receptors; Ca^{2+}-permeable channels (localized at endomembranes) such as InsP3R (a putative Ins3P receptor), a putative ryanodine receptor activated by cyclic ADP-Rib (cADPR), and nicotinic acid adenine dinucleotide phosphate-activated channels (ER), a SV channel, a slow activating vacuolar channel, and a vacuolar voltage-gated Ca^{2+} channel. All these transporters participate in a tremendously complex regulation of Ca^{2+}, whose interactions still need to be clarified. It has been shown that Ca^{2+} flux, through rat cortical neuron L-type voltage-gated Ca^{2+} channels that possess calmodulin binding domains, can lead to nuclear cAMP response element binding protein phosphorylation via a mitogen-activated protein kinase pathway. The calmodulin binding domain was shown to be critical for conveying the Ca^{2+} signal to the nucleus. This suggests that a calmodulin binding region near the channel pore may serve not only to effect channel activity regulation, but also to relay information carried by Ca^{2+} influx specifically from that channel to downstream elements. It is therefore tempting to speculate a downstream signaling role for the calmodulin binding domain of CNGCs [3]. Given the variety of plant calmodulin isoforms [4], the potential for channel-specific signaling pathways becomes greatly significant.

When considering a single cell, the involvement of Ca^{2+} in stimulus–response coupling raises the problem of how a single messenger can communicate information for specific responses to a wide range of different stimuli [5]. For example, both auxin and ABA can lead to an elevation of Ca^{2+} in stomatal guard cells [6]. However, auxin leads to stomatal opening and ABA leads to stomatal closure. The question of specificity becomes more complex when factors such as acclimation to prior stimuli are considered. For example, a previous history of drought or cold stress can significantly affect the subsequent Ca^{2+} response of *Arabidopsis* seedlings, which may involve changes in the relative contributions of different cellular Ca^{2+} sources to the overall Ca^{2+} signal [7]. Therefore, a fundamental question is whether, in addition to their maintenance functions, any of these efflux pathways help mold the dynamic form of calcium waves, and if in this manner they help define the information encoded in the signal. If efflux–influx is subjected to regulation, then elucidating the signals that control these efflux–influx systems will be equally important in identifying the signals that open various calcium channels, especially when the stimuli trigger antagonic responses.

The phytohormone abscisic acid (ABA) has been implicated in the control of a wide range of essential physiological processes, including seed development and plant adaptation to environmental stresses. For example, ABA regulates many primary water deficit responses [8–10]. Promoter deletion analyses of ABA responsive genes from different plants showed that a conserved sequence motif (ACGT), related to G-box, functions as an ABA-responsive element (ABRE) [11]. A wheat leucine zipper protein, EmBP-1, was shown to bind to the CACGTGGC sequence in the wheat Em gene [12], and different flanking regions for ACGT motifs responsive to ABA were reported [13]. It is expected that ABA-inducible promoters consist of several elements since a single element is not believed to provide sufficient specificity for the transcription regulation of eukaryotes [14]. Auxin, however, promotes a variety of growth and developmental responses, including cell extension and cell division [15,16]. Multiple auxin response elements (AuxRes) have been found to confer promoter inducibility, although the TGTCTC motif and the TGA box (or Hex-like) have been identified as being important *cis*-acting regulatory elements [17–19]. It is known that ABA accumulates during drought stress in tomato leaves, and also that changes accelerated by ABA are similar to those induced by salt stress [20]. A previous work revealed a mRNA induction of a Ca^{2+}-ATPase tomato gene (*LCA1*) by salt [21]. Following these findings a set of experiments was carried out to determine whether *LCA1* is also inducible by ABA. Since both antagonic phytohormones ABA and indolacetic acid (IAA) were capable of increasing cytosolic concentrations of Ca^{2+} ($[Ca^{2+}]_{cyt}$), the induction of *LCA1* by these hormones is also tested. How do both antagonist stimuli affect the Ca^{2+}-pumps?

Materials and methods

Construction of chimeric genes. A *Bam*HI/*Eco*RI fragment of pBI101 (Clontech, Palo Alto, USA) containing a β-glucuronidase (GUS) gene and a NOS polyadenylation site have been cloned into *Bam*HI/*Eco*RI ends of $pBSK^+$. A linker *Spe*I was introduced after *Hin*dIII digestion, and the production of blunt ends into a pBI101 vector containing the promoter of *LCA1* cloned into the *Hin*dIII/*Bam*HI ends. Plasmid 100 is the $pBSK^+$ vector (plus the GUS gene and NOS terminator), harboring a *Spe*I–*Bam*HI genomic fragment containing 2991 bp of *LCA1* promoter. A series of 5′ deletion mutants of the chimeric genes were prepared from *Bst*XI (filled with α-phosphorothioate nucleotides) and *Spe*I digestions of plasmid 100 in order to define sequences capable of conferring a responsiveness to either ABA or IAA by using the "Erase-a-base" system (Promega, Madison, USA). Chimeric genes were sequenced as described below.

Isolation of protoplasts. Seeds of *Lycopersicon esculentum* VF36 were surface-sterilized by a 20 min immersion in 1% sodium hypochlorite. After a thorough rinsing with sterile distilled water, seeds were germinated in a "Plant Con" (Flow laboratories, McLean, Virginia, USA) containing 100 ml TM-1 medium [22]. The plants were kept in a growth chamber at 25 °C, 16 h light, 8 h dark, at 4500 lx. Three to 5

days after germination, the roots were cut-off along with a portion of the hypocotyl, and the excised shoots were then transferred to a new "Plant Con" containing 100 ml TM-1. After 3–4 weeks, the containers were placed in a dark chamber at 10 °C for 1 day. The leaves were cut into small fragments (0.5 cm) and placed into 30 ml enzyme solution containing Suc 0.4 M, KH_2PO_4 0.2 M, KNO_3 1 mM, $MgSO_4{\cdot}7H_2O$ 1 mM, KI 1 μM, $CuSO_4{\cdot}5H_2O$ 0.1 μM, $CaCl_2{\cdot}2H_2O$ 10 mM, Mes 51 μM, 1% (w/v) cellulase R10, and 0.2% macerozyme R10 (Yakult biochemicals, Japan), pH 5.8, for 16 h at 25 °C in the dark. Filtered through a stainless-steel mesh (60 μm), the protoplasts were recovered from the surface after centrifugation at 50*g* for 12 min. They were washed in W-5 [23] and resuspended in electroporation buffer (0.3 M mannitol, 70 mM KCl, and 5 mM Mes, pH 5.8) at 2–5 × 106 cells/ml.

Transient expression assays. Plasmids were purified by CsCl/ethidium bromide density-gradient centrifugation [24]. Electroporation was performed as [25], in a single discharge of 650 V/cm, using a 125 μF capacitor, in the presence of 15 μg/ml DNA and 60 μg/ml salmon sperm DNA as a carrier. After transfection, samples were incubated at 25 °C in the dark, and either contained no ABA, no IAA, or with either 10 μM ABA, or 20 μM IAA, harvested 16 h later, and assayed for GUS, as described in [26]. The protein concentration in plant extracts was determined by the dye binding method [27], a kit supplied by Bio-Rad (Hercules, California, USA) Laboratories. The enzyme β-glucuronidase (G1512) and saccharic acid-1,4-lactone (S0375) were purchased from Sigma (St. Louis, MO). 2,7-Dichlorofluorescein diacetate was from Molecular Probes (Eugene, OR). Protoplast slides were flooded with a solution of fluorescin diacetate (FDA; 0.02 g FDA/10 ml acetone in 2 ml of 10% Suc) for 5–10 min.

RNA gel blot analysis. Total RNA was isolated from *L. esculentum* VF36 roots, as described by [28], and poly(A)$^+$ RNA was purified by oligo(dT) affinity chromatography using Oligotex beads (Qiagen, Chatsworth, CA) and then quantified spectrophotometrically. Two micrograms of poly(A)$^+$ RNA was size fractionated by electrophoresis in a 1.2% formaldehyde agarose gel [29] and transferred to a nylon membrane (Hybond-N), as described by [30]. Membranes were hybridized (50% (v/v) formamide, 1× SSC, 1× Denhardt's solution, 0.1% (w/v) SDS, and 0.1 mg/ml base denatured salmon sperm DNA at 37 °C for 12–14 h to radiolabeled oligo probes [γ-^{32}P]dATP (Dupont/Nen Boston, MA, USA). Radiolabeled oligo probes were obtained by labeling 5′ ends [24]. The oligo DNA fragment, which encoded a specific region of the LCA genomic DNA, LCA4702 (5′-ATCACTAT TAATTGATGTACCATAAAGGACATGAAACAAC-3′), was used to probe the *LCA1*. Following hybridization, the membranes were twice washed in 1× PSE 5 min at 32 °C and twice washed for 10 min at 30 °C.

Sequencing. All sequencing was carried out by means of the dideoxynucleotide method [31].

Results and discussion

LCA1 is induced by ABA in tomato protoplasts

To dissect the elements involved in the regulation of the *LCA1* gene, 5′ flanking fragments from the *LCA1* genomic clone were transcriptionally fused to the β-glucuronidase (GUS) reporter gene. The *LCA1* root-transcripts were previously shown to be induced by salt [21]. Therefore, a homologous transient assay system using *Lycopersicum sculentum* VF-36 mesophyll protoplasts was carried out to determine the ability of different 5′ sequences to activate the GUS gene in response to ABA. The GUS expression from the 35S promoter was not significantly affected by ABA or IAA (data not shown) under experimental conditions. This indicates that the GUS transcript produced in the experiments was relatively stable in either ABA- or IAA-treated cells. Therefore, the effects of the hormone-responsive promoters were mediated by 5′ upstream sequences and resulted from differences in transcription rates. After an uptake of the chimeric constructs into tomato protoplasts, the protoplasts were incubated in either the presence or absence of ABA and then assayed for GUS activity. The protoplast uptake was investigated after 16 h (in some cases 20, 24, 48, and 72 h were also tested). The expression of GUS driven by a promoterless construct was determined as a negative control. The synthesis of GUS was induced 4-fold following ABA treatment (Fig. 1A). Measurements of cellular Ca^{2+} have shown that plant cells maintain levels in both the cytosol and nucleus, which are 3–4 orders of magnitude lower than the levels in other cellular compartments (see Introduction). A promoter deletion containing 1699 bp of the *LCA1* promoter (construct 80) was responsible for almost all the ABA inducibility (Fig. 1A). Construct 70 still retains some inducibility while construct 40 shows no response to ABA stimuli. Therefore, the sequences which lead to promoter activities inducible by ABA are located at −1699 to −1363. This promoter region contains an ABRE element (ACGT), located from −1577 to −1574 bp from the putative translational start. *LCA1* produces two major transcripts in roots and one major transcript in leaves. The presence of two main transcription starts at a distance of 1320 bp from each other [32], by situating this motif at −185 bp upstream of the flanking transcription start. Most promoters of ABA-inducible genes contain ACGTGGC motifs within 300 bp upstream of the transcription start sites [33]. No other ABRE elements, no coupling element 3 (CE3, AC GCGTGTCCTG), no coupling element 1 (CE1, TGC CACCGG; [34]), no dehydration-responsive (DRE, TACCGACA), no DRE/CRT (C-Repeat, A/GCCG AC; [35] nor any C-repeat (TGGCCGAC; [36]) was found in the promoter region (Fig. 3). A coupling element A/GCGT [33] is located 215 upstream of the ABRE sequence. However, there is no apparent implication of this element in ABA response under the conditions tested.

LCA1 is induced by auxin

Although ABA and IAA have divergent effects on cytosolic pH (pH_{cyt}), IAA decreases while ABA increases the pH_{cyt}, both growth regulators increase $[Ca^{2+}]_{cyt}$. Does this increment mean that IAA also induces *LCA1*? To test whether the *LCA1*-promoter is controlled by the auxin hormone IAA, a series of assays using the same experimental system were carried out. From a range of 2–200 μM IAA, the highest induction level was found to be at 20 μM (data not shown).

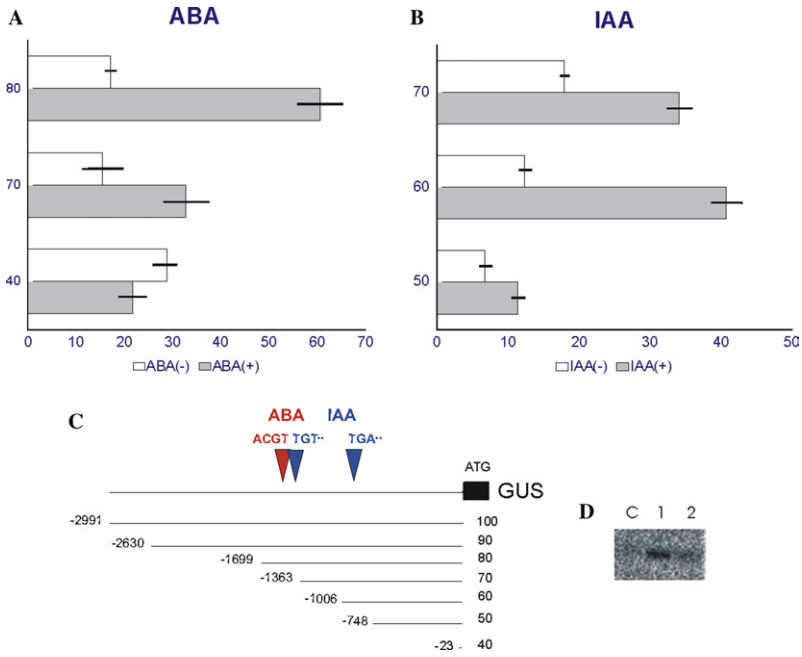

Fig. 1. Transient GUS expression in tomato protoplasts electroporated with chimeric constructs, and IAA-induced RNA blot. (A) Ten micromolar ABA. (B) Twenty micromolar IAA. (C) Deletion map, showing ABRE, TGT, and TGA motif locations in the 5′ untranslated region, and all the deletions analyzed with the corresponding first nucleotide of the 5′UTR respect to the ATG. GUS activities are expressed in pmol of 4-MU produced per hour and per microgram of protein. (D) RNA gel-blot analysis of *LCA1* in plants exposed to IAA. Each line was loaded with 2 μg poly(A) RNA isolated from roots. C, no IAA; 1, 10 μM IAA; and 2, 1 μM IAA.

The transcriptionally fused sequences capable of conferring most of the inducibility were localized within deletion 60 (Fig. 1B). This region, spanning from −1006 to −748 with regard to the translational start (Fig. 3), contains two TGA sequences, that, when removed, cause the *LCA1*-promoter to become almost unresponsive to IAA. There is also a TGTCTC sequence located at −1430 from the putative ATG, that does not play the most significant role under the assay conditions carried out in this work. This induction of the *LCA1* expression, triggered by IAA and detected by using GUS, was confirmed by Northern analysis (Fig. 1D). *LCA1* is therefore induced by IAA, increasing mRNA levels when roots were exposed to different IAA concentrations. The highest level of mRNA synthesis was found at 10 μM IAA.

Measuring the GUS activity in tomato protoplasts revealed significant endogenous interferences

When protoplasts were assayed to measure the fluorescence emitted by 4-methylumbelliferone (4-MU) for the different deletions of the *LCA1* -promoter, interfering values accounted for some of the total fluorescence quantified. The signal-to-noise ratio of the detection method was evaluated. The promoterless-GUS fusion, when electroporated in mesophyll tomato protoplasts, exhibited endogenous levels of fluorescence. Non-electroporated protoplasts, therefore containing no chimeric glucuronic protein and no modifications due to the electrical fields, exhibited similar absolute values of fluorescence (Fig. 2A). In order to clarify whether the detected activity was due to fluorogenic compounds other than 4-MU, a kinetic determination was performed in each of the experiments carried out in this work. To establish the optimal window of measurements for each particular assay, a set of kinetics was prepared from a few minutes to 2 or 3 days. The GUS-derived fluorescence incremented with time, whereas the relative fluorescence due to other fluorogenic compounds remained relatively constant (Fig. 2B). However, neither the use of REG (resorufin β-D-GlcUA), an alternative substrate of GlcUA which emits at a wavelength of 465 nm, nor the use of 20% methanol, nor alternatively the use of 0.3% formaldehyde, completely eliminated the basal level of fluorescence observed (data not shown). When the mixture of synthetic ABA was substituted by natural ABA, no significant change was observed in the pattern of expression. In general terms, the use of REG as a substrate resulted in less absolute values of activity than the use of 4-methylumbelliferyl β-D-glucuronide (MUG), indicating less interference of fluorogenic compounds and a higher reproducibility, as expected (data not shown). Different dilutions of commercial GUS enzyme were included in all the assays to determine the linearity of the fluorescence measures carried out in the experiments. The use of 3 mM D-saccharic acid 1.4-lactone, a substance which is metabolized to D-glucaro-1.4-lactone,

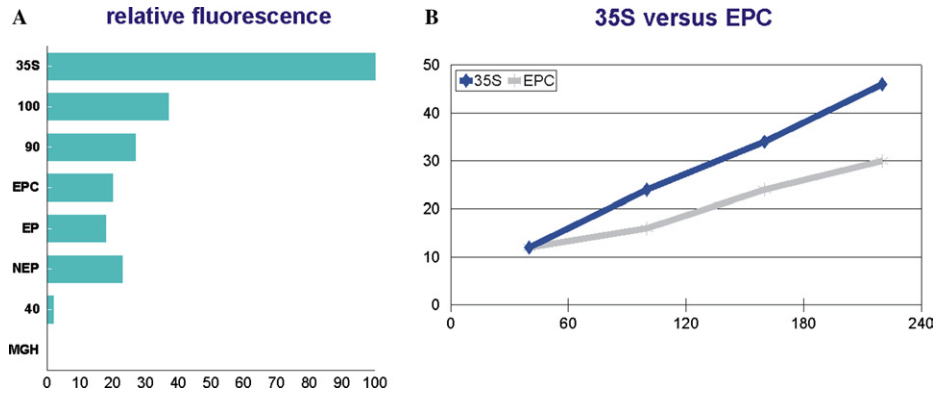

Fig. 2. Evaluation of interferences in the determination of GUS-expression in tomato protoplasts. (A) Relative fluorescence of the different elements included in assays of GUS expression. The quantities determined for the deletions of *LCA1*-promoter are expressed with respect to measures obtained for the construction of 35S-GUS (35S). Values are compared in percentage of activities which are expressed in pmol RE/μg protein h, except for EPC, EP, HEP, and MGH. RE (resorufin) is the product of REG. The numeration of *LCA1*-deletions is relative to promoter start as in Fig. 1C. EPC, electroporated protoplasts plus carrier; EP, electroporated protoplasts; NEP, non-electroporated protoplasts; and MGH, MUG hydrolysis. The numbers are the average of at least three experiments with similar results. (B) Kinetics of 35S and EPC. Results are the average of two experiments with similar results.

AAAAATTATACATTTTATTTTGTATAATTAGTGTTTGATATAAAGAGAGA -2042
GAGAGAGATTTGCATATTATTTGTATATACAATCTCTCTCGCTTTATACA -1992
AATATAAATACAATTTATATATTTGTATTTGTATAAAGTGAAAGAGACGA -1942
TGGAGAGTAGCGAGCGAGATTAAAAAAGAGTGGCGAACGAGATATGCCGT -1892
AAATTAGAATTAAATGAAACTGTCATTATAACATTTATTTTGAATAAATA -1842
ATTTTGATATAATACACAATTTTCTTAAAAAGCAACGAGATAATGTAGCG -1792
TAGAGTTTCAAATCTAATGTTCCCTCCTTTACTTACTCCCCTCCAACTCA -1742
TTCATAAGTTAATTATCACTCAGCTATATTTTACTGAAATAACATTATAA -1692
GATTGATTTTGAAAACACATTCAACTTTACTCAAATATACATAGAAGTCA -1642
ATATATATATATATAACTTTGCTAAATATCTCAATTAGTCACTAAACAAT -1592
TCTCTTGTAACCAAACGTCATTCAACATTGCTAATCTTTTTATTCCTCCT -1542
AATGACTTCCTATATTTAAATAAATAAAGTTGAGTATATATAAAAAATAA -1492
TTTAATGATTTATAATTGTATATAATATAGTATATACAGTTTACGCGTAC -1442

GGCTGTCTACTTGTCTCCGGCTACTACCACATAAAAGTCAAACTCCAATT -1392
TTAAGGACATTATGGTAAATTCATTATCCACTTCGATTTTATAATCCCAA -1342
CTGTTTCAAAATCTTATTTTTGTACAAGTTTTTCCGTCTCTTTCTGAAGA -1292
GTAAAAACAGTTATTCCATTTCTAATTTCAAGCAAGTGTTGTTCTTTCTT -1242
GTTTTTCACTAAAAATTATTACTTTATTGAATGCTTTTTTCAAGCAAAGA -1192
AATTTTTTGAGCCGTAAGTTTCCAAGAATTTTCTTTCTCATCATATTATG -1142
TCCGATCTGTCAGTTACTTTCTCTCAAAGCCACTGCTTTTGTCCCTTTAT -1092
AAGAGGTACTACAACATTTCCTCAACTCATTGTATTCCTTTTTGTCACTT -1042
CTTGATTTTGGGATTTATGGTTGTTGCTTCCTTGCAATTGCTAAAGTTTC -992
AATCTTTTTTCTTTTATTTTTTCATGTGGGTACTGTATTAATGACATGAT -942
CTGTTTTGATTCTTCTTTATGTGACCGTTATTCTTGTTGTATATGTATAA -892
AGTTGTTTGCTTTGCATGTTTTTTAGTTTCCATATTGCTGTATACAAGTT -842
TGTCGGGTTGTCGATTTTGTTCCTTGATACTTTAATGCTGCTTGGATGGC -792
GTCCCTGTTTTGCTTTTAATTTACTCGTGGTTCACAATTTGTTAGGTCTG -742
TCATAAAAGCTGTCAATTAAAACTCAGTAGAGTTGTAGTTGGAGACACCC -692
CACAAAGTTAGGGTAATGTCTGTGTACCTCATACCCTCCTTCTCCGGACT -642
TATCCTGGGTATGTTGTTGTTATGGTAGTGTTTCTAGTTGGAACTTGTTT -592
TTTTTTTTTTTTTACCTATTATTATGTGAATTTTCTGTTATTTGGTCTTC -542
ATGTTTGCTCTGTGTGTTATTTGGTTAGGGCAAAGTCTAATGATATAGTT -492
TCTAGCTTCAGTGATGTTAATTTAGCTTTCCTTCTCTATTATTTTTGCTT -442
AAATTTTATGGTTTTAGTTTTGCACACTAATAATACTACTCCCTCTGTCC -392
AATTTTAAATGGAGCAATTGGAATTTTGAGATTCAAACTTATAAAATTTC -342
AGTGTGATTTTTTTTATATGGCAATTTCAGTGTGAATTCAAATATAGAAG -292
TTTTAAGTTTTTTGAAAATGAAATTTGTATATCTGAAAGCTACTCACAAG -242
TACTAAAATAAATCACAATAATTAACAATTTAAAATATTTAATTGGCATA -192
TAAAGAAATTCCGGTTAAAGAAAAACTTGTTTGACTCTCAAAATTCCAAT -142
TGTGCCACATAAATTGGGATGGATGGAGTATATTAGTACTTCTAAGCTCA -92

AACTGTTAATACTTGTATCTCTTTTCAGGGAATGAGATTATTGAACATAA -42
TTTCTGTGGAATTTAAGTTAAGACTGAGGATTTGATTTGCGATGGAAGAA

Fig. 3. Consensus sequences responsive to ABA and IAA. 5′ UTR sequence of *LCA1*. The ABRE element (red) and the elements responsive to IAA (blue) are represented in colored boxes. The first transcribed nucleotide (T) is in bold letters. Other important elements in the control of gene expression are in bold (CAAT or G-rich sequence), underlined (AGGA) or in transparent boxes (TATA box). In red: coupling element. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

an inhibitor of the enzyme β-glucuronidase, caused a drastic drop in the increment of MU concentration. This fact was observed for non-electroporated protoplasts and for protoplasts expressing *LCA1* deletions, by decreasing the level of transient expression in a $39 \pm 4\%$ after a 1-h inhibition. Non-electroporated protoplasts decreased the level of transient expression by $44 \pm 1\%$. The basal level of fluorescence might be explained as a result of the synthesis of fluorogenic compounds and by an endogenous GUS presence, both of which were most probably caused during the preparation of protoplasts, since when GUS activity was assayed in transgenic plants of tomato this endogenous activity was not detectable (Wimmers, personal communication). The standard deviation of the fluorometric values obtained for the same deletion electroporated twice in the same experiment was 0.7%. Fluorescein diacetate was used to determine the cell viability of protoplasts after electroporation, estimated to be around 70% of the total number of protoplasts. An addition of 0.02% NaN_3 to prevent microbial growth as well as 100 μg/ml BSA to stabilize enzyme activity was carried out for long assays. Fluorescence of the extract alone was also determined (Fig. 2A) in order to discard the fact that initial levels of fluorescence are often contributed by traces of pre-hydrolyzed MUG in commercial substrate preparations or of coumarins in the extract. The fluorescence increment observed for non-electroporated protoplasts, electroporated protoplasts, and protoplasts electroporated with carrier and substrate hydrolysis (MUG) in the reaction mixture, with regard to the average values of construct analyzed, was inversely proportional to the reaction time employed in each assay. For example, the expression of GUS controlled by the 35S promoter produces the following activity (pmol MU/μg protein h): 300 determined in protoplasts of rice, and 222 determined in protoplasts of petunia. In tomato protoplasts, this activity reached 60 pmol MU/μg protein h.

Endogenous fluorescence

The utilization of protoplasts may lead to a rapid and accurate method to investigate gene expressions without any need to either obtain or to wait for transgenic plants. Transient expression studies conducted in protoplasts made of species different to that housing the gene object of investigation may be the origin of inaccurate conclusions since their transcriptional and translational machinery can be considerably different. The motifs responsive to one stimulus may not work in a different species, and those that may produce a response may not be functional in the original species. It is for this very reason that an emphasis was placed upon the expression of a tomato gene (*LCA1*) in protoplasts, specifically made from tomato (mesophyll cells of leave tissue), in this report. It is demonstrated that the GUS-reporter fusion analysis under the conditions assessed in this work produces significant levels of the endogenous signal. This signal is in part due to fluorogenic compounds present in the media, but also due to internal β-glucuronidase activity that is more likely to be caused by the protoplast preparation process, probably when the degradation of cell wall takes place. Detoxification of exogenous and endogenous cytotoxins is a four-phase process. In phase II (conjugation), the activated derivate (produced in Phase I) is conjugated with GSH (animals and plants), Glc (plants), sulfate (animals), and with GlcUA (animals and possibly some plants), by means of UDP–glucuronyltransferases in the latter case. Kinetic studies in rye suggested that the vacuolar glucuronide transport system is constitutively expressed throughout primary leaf development [37]. In contrast to the mesophyll cells, the epidermal layers do not contain glucuronated flavonoids, rather glycosylated flavonoids [38]. It is possible that the mesophyll protoplasts obtained from tomato may have and may activate a similar transport system. A limited number of glucuronated secondary compounds have been reported in plants [39]. For example, R1 (luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide) is a flavone glucuronide found in rye that is thought to be exported from the vacuole, and a turnover is precisely started by a specific β-glucuronidase located in the apoplast [40,41]. The metabolism of these flavone glucuronates starting from luteolin is sequentially catalyzed by three anabolic cytosolic UDP–glucuronate: flavone–glucuronosyltransferases. This is followed by vacuolar storage via an unknown mechanism, and this may also involve a subsequent degradation of the luteolin triglucuronide initiated by a specific β-glucuronidase located in the cell wall. Since non-electroporated protoplasts were sensitive to a specific inhibitor of β-glucuronidase, it is tempting to suggest that the activity of enzymes required to prepare protoplasts (cellulases, macerozyme) produces such a toxin, and that they are processed as xenobiotics by the cell, by increasing the production of GlcUA, perhaps fluorogenic compounds, and of the GUS liberation from the cell wall. In fact, when the gene β-glucuronidase of *Escherichia coli* was compared with the EST sequences deposited in the tomato GenBank, a 97.10% nucleotide identity was found for a cDNA fragment (EST588853), that strongly supports the hypothesis of an internal β-glucuronidase presence (Fig. 4).

One single copy of ABRE is responsive to ABA

5′ deletions of *LCA1*-promoter fused to a GUS reporter gene identified a 337 bp fragment as the promoter sequence that is capable of conferring most of the responsiveness to ABA (Figs. 1A and C). This deletion contains a previously characterized *cis*-acting element,

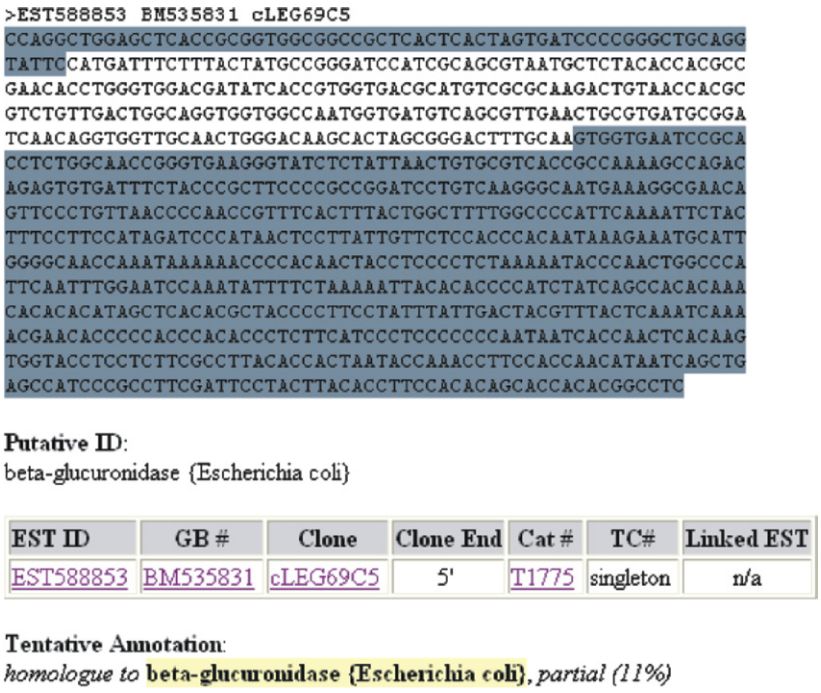

Fig. 4. GUS homology to tomato EST sequences. Comparison was carried out by using TIGR (The Institute For Genomic Research) software applications in a tomato breaker fruit library (pericarp tissue). GB#s are linked to GenBank accessions. Clone End describes which end of the cDNA was sequenced. Cat# links to a report for the library from which this EST was derived. Low quality, vector, and polyA/T regions (trimmed from the EST) are shown in gray. ID, identity, Sim, similarity.

whose consensus sequence (aACGTca) presents three atypical characteristics: it is unusually distant from the putative translational start (−1577 bp), it contains a purine nucleotide upstream of the consensus sequence, with a C instead of a G immediately after this string. Nevertheless, the ABRE motif is located 185 nt upstream of the start of the largest mRNA-transcript and this fact is usual. Single copies of ABRE have been found to require a *cis*-acting, coupling element to achieve ABA induction. The promoter region of *LCA1* meets this requirement: it contains one single copy and a coupling element AGCGT, that is located 215 upstream of the ABRE sequence. However, when this coupling element is absent, the *LCA1*-promoter induction is still present under the conditions used in this work. The induction of this Ca^{2+}-ATPase by ABA, in total accordance with the previously found *LCA1*-mRNA induction by salt, may represent an increased capacity of intracellular Ca^{2+} sequestration after ABA had triggered the increase in the cytoplasmic Ca^{2+} levels, thus restoring the steady-state levels of $[Ca^{2+}]_{cyt}$. The Ca^{2+}-ATPases are known to pump Ca^{2+} from the cytosol by decreasing intracellular Ca^{2+} concentration, which in turn can either activate or inhibit the expression of many genes. The fact that the increment triggered by ABA equals the ratio $[Ca^{2+}]$ in organelles/$[Ca^{2+}]_{cyt}$ (or in the nucleus) might indicate that the extrusion from organelles (and/or in the nucleus) is proficient enough to reach the cytosol/organelle equilibrium, and further restored by the TN-Ca^{2+}-ATPase in the vacuole. The particularities observed regarding the context of the ABRE sequence found in the *LCA1*-promoter may be imposed by the specific Ca^{2+}-ATPase functions. The fine-tuned regulation of one enzyme that is crucial for Ca^{2+} depletion after many stimuli may perhaps require certain specific peculiarities that can help to link a general responsiveness (such as one generic gene controlled by the signal) to a hormone in a singular manner since Ca^{2+} responds to many stimuli (some antagonist), and more importantly, in a complex rapidly changing message (Ca^{2+} waves). Although the utilization of protoplasts produces high benefits, this technique also shows disadvantages. Since the promoter of the gene investigated is not integrated in the genome of the host organism, no chromatin modulation is then possible. Therefore, *LCA1* may be far from its ''standard conditions'' of regulation. In fact, the studies of the chromatin structure of ABA responsive genes indicate the importance of the transcription induction either by co-activators or by the phosphorylation/dephosphorylation of transcription factors. The promoters of genes inducible by ABA are seen to be in an open chromatin structure, and the nucleosomes are probably absent or modified. For instance, the constitutive transcription factor binding to the ABRE and to other elements in the *rab17* and *rab28* promoters in leaves suggests that induction by ABA is regulated by the modification of protein–protein contacts or by posttranslational

modifications. Besides, it has been reported that in transient expression assays the requirement for the signals acting in combination is at least partially relaxed [42]. The protein kinases and phosphatases that participate in ABA signaling could regulate constitutively bound transcription factors on ABA-inducible genes either by phosphorylation or dephosphorylation. For instance, the *Arabidopsis* GF14 protein, which is associated with the ACGT-element binding factors, has a homology to proteins that regulate the Ca^{2+}-dependent protein kinases activity in mammals [43]. This protein could thus participate in the control of ABA-induced phosphorylation.

IAA induces LCA1 expression

LCA1 is also induced by IAA. Although the induction of the *LCA1* expression by both ABA and IAA is not unique, it constitutes an uncommon way of regulation for a single gene in plants. Unlike the loss of the auxin inducibility seen for GH3 promoter when this sequence was deleted [17], the IAA inducibility is still present when a conserved TGTCTC element found to be 38 bp upstream of the distal transcription start is removed. Similar to the case of ABA, it might be possible that the general rules of IAA inducibility observed for a cluster of genes may not be necessarily true for *LCA1*. It is feasible that this gene, which is involved in the critical process of controlling intracellular calcium levels, is under the same transcriptional control in response to auxin, although distinctly modulated. A sequence spanning from −1006 to −748, relative to the putative translational start, is responsible for most of the IAA inducibility. This sequence contains two TGA elements surrounded by AT rich sequences (Fig. 3). TGA-like elements are able to confer inducibility in some auxin-responsive promoters, as previously reported [44]. This *LCA1* inducibility by IAA has also been confirmed by Northern analysis using a specific *LCA1* probe (Fig. 1D). Strikingly in the root tissue, only the vacuolar Ca^{2+}-ATPase is induced by IAA. For some reason, the $[Ca^{2+}]_{cyt}$ after the increment produced by IAA has to be restored to the steady-state level, without any increment in the amount of the synthesized PM-isoform being needed. Perhaps only when the Ca^{2+} has to be extruded outside the cell to deliver a message to the rest of the tissue, or when it is exported to a complete homeostasis balance, does the PM Ca^{2+}-ATPase exhibit an important role of participating in this extrusion. By contrast, when an internal message has to be distributed without any need of external delivery, the TN Ca^{2+}-ATPase has to be induced to restore the steady-state balance of $[Ca^{2+}]_{cyt}$.

Why ABA and IAA together?

The cross talk between different signaling pathways is widely present in plant cells, especially on hormone regulated pathways. Although ABA is involved in plant growth inhibition, and auxins are involved in plant growth development, both IAA and ABA finally increase $[Ca^{2+}]_{cyt}$ [45–47]. ABA raises the pH prior to the increase of $[Ca^{2+}]_{cyt}$, however IAA lowers the internal cellular pH by acidification, via the H^+ pumping of an IAA-induced ATPase-isoform [48]. Since both hormones increase $[Ca^{2+}]_{cyt}$in the end, and since the Ca^{2+}-ATPases may restore their level to the steady-state concentration, the previous evidence is consistent with the demonstration that IAA as well as ABA induces *LCA1*, as reported in this work. Similarly, other hormones such as GA stimulate the Ca^{2+} influx, by triggering an increased expression of rice ER Ca^{2+}-ATPase in this case [49], indicating that this gene family of the Ca^{2+}-ATPases is regulated by multiple signaling pathways. The list of genes regulated by ABA and IAA is continually on the increase. Results of expression profiles in rice shoots using cDNA macroarrays showed that in fact a group of clones, regulated by ABA, were also regulated by IAA. For example, polyubiquitin 6, cytochrome P450, MADS box transcription factor, a protein associated to PSII, etc [50]. Long-distance signaling under stress has been associated with the transport of ABA from the root to the shoot, also in the cytokinin/auxin transport, or through ethylene. The analysis of mutants with altered ABA responses indicated regulatory roles for the loci AXR2 in resistance to auxin and ethylene [51]. These may indicate the complicated interactions of signal pathways with different hormones. Salt stress and ABA rapidly induced *osgstu3* in rice roots, whereas *osgstu4* exhibited a late salt stress and no ABA response. Salicylic acid, jasmonic acid, and the auxin K-naphthalene acetic acid triggered *osgstu4* and *osgstu3* expressions. In addition, the RsMnSOD (a cDNA clone for a mitochondrial MnSOD isolated from a cDNA library derived from seedlings of the small radish, *Raphanus sativus* L.) expression was strongly induced by osmotic stress, moderately induced by phytohormones such as ABA and IAA, and not induced by xenobiotics, other than cercosporin [52]. Which are the mechanisms for this ABA and IAA gene regulation? Do they have common pathways? One reasonable possibility points to the inositol cycle of calcium signaling. This is a main transductional activator of many signals, acting as a sensor and a signal transductor at the same time in most cases. Very recently, DNA chip-based analysis under hormone treatment has shown that for example, IAA treatment, associated with plant growth, triggered the up-regulation of the PLC and PIPK family members. In other cases, a specific member of one of the families (PLC8) was up-regulated following treatments with IAA, ABA, mannitol, NaCl, Ca^{2+} and with MeJA, while another member of the family (PLC5) was suppressed by IAA and ABA treatments. Besides, IAA and ABA both altered PI signaling. The expression prof-

ilings of the PI pathway genes were similar following treatments with ABA and IAA, Ca^{2+}, and mannitol [53]. Thus, ABA and IAA alter the concentration of one specific intermediate of the inositol cycle. This may cause the incremented concentration of the next components of the cycle, increasing the production of IP3 at the end, which in turn promotes the Ca^{2+} efflux from internal stores to deliver the specific message containing adequate instructions. At the same time, for example, ABA induces the Ca^{2+}-dependent protein kinases [54]. As already mentioned, the protein kinases (in combination with phosphatases) participate in ABA signaling by regulating constitutively bound transcription factors on ABA-inducible genes by phosphorylation or dephosphorylation. Finally, once the message has been sent while ABA- and IAA-genes have been induced, an excess of cytosolic Ca^{2+} might be deleterious to the cell, by triggering apoptosis, $[Ca^{2+}]_{cyt}$, for instance. Therefore, $[Ca^{2+}]_{cyt}$ is subsequently driven back to the steady-state levels by enzymes like the vacuolar Ca^{2+}-ATPase, that was previously induced by ABA and/or IAA.

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