Identification and subcellular localisation of *INT7*: A novel tomato (*Lycopersicon esculentum Mill.*) fruit ripening-related and stress-inducible gene.

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ABSTRACT

In order to identify novel ripening-related C_2H_4-dependent components, a yeast two-hybrid interaction screen have previously been employed in which NR cDNA, a key C_2H_4 receptor gene whose expressing is induced during ripening, has been used as bait. This screen has identified a clone corresponding to interacting protein 7 (LeINT7), through its specific and strong interaction with the NR receptor (Alexander et al., unpublished work). In this work, our objective was to identify the corresponding NR-interacting gene and subsequently characterize its expression response to various stress treatments, as well as unravelling its subcellular location in the cell. By sequencing and plant data base interrogation, LeINT7 was found to be a small gene with an ORF of ~243 bp encoding a protein composed of 77 aa that shares no sequence homology with any known gene. Northern analyses demonstrated that LeINT7 gene expression is up-regulated in response to various stress signalling molecules such as salicylic acid, abscissic acid, jasmonic acid, nitric oxide and salt, implicating Int7 in biotic and abiotic stress signalling responses. A chimeric construct in which LeINT7 is C-terminally fused to the green fluorescent protein (GFP) was generated. Thus, 35S::LeINT7::GFP-containing constructs were transiently expressed in both tobacco leaves and onion peels via microprojectiles bombardment. Subsequently, confocal laser microscopic examination of bombarded tobacco and onion tissues revealed that the expression of GFP-LeINT7 was observed predominantly in the plasma membrane, compared to the location throughout the cell observed with the control GFP construct alone.

Keywords: *Ethylene, fruit ripening, subcellular localization, tomato.*

INTRODUCTION

The gaseous phytohormone ethylene (C_2H_4), despite its structural simplicity, plays a critical role in the regulation of developmental programmes throughout the plant life cycle and serves as a major response mediator to various environmental signals. Fruit ripening and biotic/ abiotic stress signalling are among the C_2H_4-regulated processes (Morgan and Drew, 1997; Bleecker and Kende, 2000). Fruit ripening is a genetically controlled complex multi-event, associated with enhanced C_2H_4 biosynthesis, which can be described as the summation of biochemical and physiological changes leading to a ripe phenotype conferring a characteristic texture, colour, taste, flavour and aroma of the fruit flesh (Alexander and Grierson, 2002). Fruit development occurs in five stages, including organogenesis, expansion, maturation, ripening, and senescence. In climacteric fruit, such as tomato, ripening is preceded by a dramatic increase in C_2H_4 evolution, which remains at basal levels prior to the onset of ripening (Klee, 1993; Lashbrook *et al.*, 1998).

The majority of components implicated in C_2H_4 perception and signal transduction pathway have been identified by a combination of biochemical and molecular genetics approaches with *Arabidopsis thaliana* mutant characterization. In *Arabidopsis*, C_2H_4 is perceived by a family of five endoplasmic reticulum (ER)–localized C_2H_4 receptors (ETR1/2, ERS1/2 and EIN4) (Wang *et al.*, 2002) that share sequence similarity with the bacterial two-component histidine (His) kinases, which consist of a sensor protein and a separate response regulator protein that function together, allowing bacteria to respond to different environmental conditions (Chang and Stewart, 1998). ETR1 is the founding member of the receptor family and has been localized to the ER membrane (Chen *et al.*, 2002). The particular physicochemical properties of the C_2H_4 gas allow it to freely diffuse through the membranes and the cytoplasm, eliminating the need for an active transporter system to deliver the ligand to its receptors in the ER. It has been suggested that the C_2H_4-binding site is located in the first two transmembrane domains of the receptor and that the binding is mediated by a copper co-factor, providing the required high binding affinity and specificity of the ethylene receptors (Wang *et al.*, 2006). Knowledge of the detailed C_2H_4 signalling pathway defined in *Arabidopsis* enables comparative analyses to be carried out in other important crop species such as tomato (*Solanum lycopersicum*), where C_2H_4 is critically involved in the fruit ripening process. In tomato genome, a family of six different C_2H_4 receptors (LeETR1–LeETR6; LeETR3 is referred to as Never-Ripe, NR) has been identified, all of which are differentially expressed in various tissues (Tieman and Klee, 1999). Transcripts encoding two C_2H_4 receptors, *NR* and *LeETR4*, accumulate to high levels in ripening tomato fruit, suggesting that these two receptors may function in C_2H_4-induced ripening (Wilkinson *et al.*, 1995; Yen *et al.*, 1995). It has been observed that in transgenic tomato plants where NR expression is reduced by antisense inhibition, expression of LeETR4 increases proportionally. It appears, therefore, that somehow the tomato plant compensates for the loss of NR by increasing the expression of LeETR4. This phenomenon, referred to as functional compensation, has not been observed in *Arabidopsis* (Tieman *et al.*, 2000; Kevany *et al.* 2007). Recently, the yeast two-hybrid approach has been successfully employed for the study of protein association and subcellular localisation of known C_2H_4 receptors (i.e. LeETR1, LeETR2 and NR) and downstream signalling components (i.e. LeCTRs) (Zhong *et al.*, 2008). Previously, NR has been utilised as bait in a yeast two-hybrid screen of a library generated from tomato fruit (Alexander *et al.*, unpublished work).

To gain more insight into the tomato ethylene signalling mechanism we have identified and functionally characterised a novel NR-interacting protein, designated LeINT7. In this work, we show that *LeINT7* transcript level increase during ripening in a fruit-specific manner as well as in response to various hormonal and stress-related factors. Moreover, experimental evidence is presented in relation to *in vivo* subcellular localization of green fluorescent tagged protein fusion with LeINT7 in tobacco and onion epidermal cells. These results are discussed in the light of the current knowledge on C_2H_4 signal transduction, and its cross-talk with other stress signalling pathways.

MATERIALS AND METHODS

Plant material

All experiments were performed using a near isogenic line of diploid *Lycopersicon esculentum* Mill. cv. Ailsa Craig (AC^{++}) plants. Fruits from selected fruit ripening mutants were also used (i.e. *Nr* and *rin*) for *LeINT7* gene expression analysis. Unless otherwise stated, plants were grown in 24 cm diameter pots in M2 compost (Levington Horticulture Ltd., Ipswich, Suffolk, UK) in growth chambers with a diurnal regime of 16 h continual light (250 mmol m^{-2} s^{-1} photosynthetic photon flux) at 23 °C followed by 8 h continual dark at 18 °C. One-week old tobacco seedlings grown under sterilised conditions were utilised for biolistic bombardment.

Plant treatments

Eight-week old *Lycopersicon esculentum* AC^{++} plants were utilised for chemical treatments. Plants were divided into two groups, eight plants each, group I taken as spray control (Sc) and group II was sprayed with one of the following solutions containing: 10^{-5} M abscissic acid (ABA), 0.2 M NaCl, $2x10^{-4}$ μM jasmonic acid (JA) or 10^{-4} M salicylic acid (SA) prepared in sterile distilled water, to which Tween X-100 has been added to a final concentration of 0.5% (v/v). Spray control plants of AC^{++} were sprayed with deionised water containing the same concentration of Tween X-100. In all cases, leaves and stems were sprayed to the drip point. For the experiment using the NO-donor sodium nitroprusside (SNP) detached AC^{++} leaves were incubated in 50 ml Falcon tubes containing 10^{-3} M potassium phosphate buffer (pH 6.5) for 12 h prior to SNP treatment to relief wounding effect. Subsequently, the incubation buffer was replaced with a solution of 10^{-3} M SNP prepared in the same buffer. In all cases, leaves were collected at specific time points, snap-frozen in liquid N_2 before stored at -70°C until further use.

Construction of GFP fusion with LeINT7 cDNA

The pGEM$^{@}$-T Easy vector, harbouring a previously cloned *LeINT7* cDNA, was used to PCR-amplify the full-length *LeINT7* cDNA using the high fidelity *Pfu DNA* polymerase. In order to generate C-terminal LeINT7 fusion with GFP, the primers *LeINT7_F* (5'-GATGGGATGCTTCGATTGCTTCTA-3') and *LeINT7_R* (5'-TGCTGCTCCCATCTGCCATTTAAG-3') were used, taking into account the replacement of the stop codon. The blunt-end *Pfu*-amplified *Int7* PCR product was A-tailed prior to its cloning into an entry vector for Gateway$^{@}$ system (Karimi *et al.*, 2002) using pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen) according to the manufacturer's instructions. Subsequently, the INT7-containing entry vector was allowed to react with the Gateway$^{@}$ vector pK7FWG2.0 (Karimi *et al.*, 2002), leading to the generation of *35S::INT7::GFP* fusion construct, employing Gateway® LR Clonase™ II Enzyme Mix (Invitrogen), according to the manufacturer's instructions.

In silico sequence analysis and database search

LeINT7 homology with known plant sequences was analysed by employing BLASTn and BLASTp (Altschul *et al.*, 1997) at the Plant Genome Database (PlantGDB) and Solanacea Genome Network (SGN) database. In order to test for the presence of localisation signals the following publicly-available programmes were employed: PSORT (Nakai and Kanehisa, 1991), ChloroP1.1, Predator, SignalP3.0, MITOPROT. For the prediction of amino acids phosphorylation sites NetPhos2.0 was utilised. Finally, for the prediction of coiled coil protein regions PAIRCOIL and COILS were employed.

Extraction, purification and analysis of plant RNA

RNA was extracted from tomato fruit pericarp tissues and other vegetative organs according to the method described by Smith *et al.* (1986).

In planta transient expression system

The preparation of biolistic microparticles was essentially conducted as previously described (Aboul-Soud *et al.*, 2004).

Confocal and fluorescence microscopy

Tobacco leaves and onion epidermal peels were transferred to glass slides and analysed. Images were collected with a Leica TCS SP2 AOBS confocal laser scanning microscope (CLSM) with an argon/krypton laser and a fluorescence microscope (Leica and Nikon). The laser lines available are 488 nm (FITC), 568 nm (TRITC, Texas Red etc.) and 647 nm (Cy5). Scans of the resulting green (from GFP) and red (from chlorophyll) fluorescence were superimposed to reveal GFP localisation.

RESULTS AND DISCUSSION

LeINT7 is a novel ripening-specific and stress-inducible gene

The NR-interacting clone was sequenced and it was shown to correspond to a ~550 bp gene with a short open reading frame (ORF) of only ~ 243 bp. (Fig. 1). The *LeINT7* ORF was shown to encode a protein product of 77 amino acids (Fig. 1). Homology search (BLASTn and BLASTp; Altschul *et al.*, 1997) of the *LeINT7* nucleotide and its deduced protein homology with known plant sequences was conducted by interrogating the PlantGDB and SGN databases. This search revealed that *LeINT7* is not homologous to any known plant nucleotide or protein sequences in the interrogated databases (data not shown). The mRNA expression profile was examined in various tomato tissues via northern gel blot assay (Fig. 2). The *LeINT7* mRNA was not detected in leaves, flowers, roots and stems. Interestingly, gel blot analysis revealed a fruit expression of that *LeINT7* mRNA during ripening, peaking at Br+7 stage (Fig. 2). Moreover, while *LeINT7* expression was not detectable in MG fruit, treatment of MG fruits with 10 ppm C_2H_4 failed to induce its expression (Fig. 2). Notably, *LeINT7* expression was blocked during the fruit breaker stage in the fruit ripening mutants *Nr* and *rin* that fail to fully ripen (Fig. 2). Hence, the obtained results (Fig. 1 and Fig.2) clearly indicate that *LeINT7* is a novel gene whose transcripts exhibit fruit ripening-related expression that is dependent upon fully functional *NR* and *RIN* genes. The *Nr* mutant cannot perceive C_2H_4 due to a mutation in the C_2H_4-binding domain of the NR ethylene receptor (Lanahan *et al.*, 1994; Wilkinson *et al.*, 1995). The mutation of the Nr^+ C_2H_4 receptor prevents ripening in tomato via C_2H_4 insensitivity demonstrates that climacteric C_2H_4 signalling plays a central role in coordinating the molecular processes required for ripening (Lanahan *et al*, 1994; Wilkinson *et al.*, 1995). Fruit from the ripening inhibitor (*rin*) mutant do not show autocatalytic C_2H_4 production (Herner and Sink, 1973) and cannot transmit the ethylene signal downstream to ripening genes due to a mutation in the RIN transcription factor (Vrebalov *et al.*, 2002). *LeINT7* transcripts didn't accumulate in C_2H_4-treated mature green (MG) fruit (Fig. 2) indicating that *LeINT7* mRNA expression occurs in a C_2H_4-independent fashion and that is could be developmentally regulated. This observation confirms previous findings that ripening-related expression is not always dependent on C_2H_4 (Vrebalov *et al.*, 2002).

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1     GGA AGA GAG AAG ATG GGA TGC TTC GAT TGC TTC TAT GGG GGC AGC   45
1                     M   G   C   F   D   C   F   Y   G   G   S     11

46    AAA AGA GAA CAA AGG AGA GAA GAA GAA CAA TTA GCC TCC GAA GAA   90
12     K   R   E   Q   R   R   E   E   E   Q   L   A   S   E   E    26

91    GCT CGT GCC AGA GCT GCC GAA GCC GCC CAA AAA AGG CAA GAA CAA   135
27     A   R   A   R   A   A   E   A   A   Q   K   R   Q   E   Q    41

136   TAT GAA AAA TCT GCC GCA GGA AGA GCA GCA CGT GCA CAA ATG GCA   180
42     Y   E   K   S   A   A   G   R   A   A   R   A   Q   M   A    56

181   GCT GCT GCC AAG CAA GCA ACG AAT GCA AAC CAG GGA GAA CCA GTT   225
57     A   A   A   K   Q   A   T   N   A   N   Q   G   E   P   V    71

226   CTT AAA TGG CAG ATG GGA TGA GCA TTA GTT CTC TTA GGT CAA CTT   270
72     L   K   W   Q   M   G   *                                    77

271   CTT TCA TTC ATT GTA CTG AGA GTT GTA GTT TGA TGT CCA ACA GTT   315

316   GTA AAT ATG TTG CTC TTA TTT AAT TTA CTG TTT TCT CCA ATC TAA   360

361   ATA TTT GTA AAC TAA AAT GAG AAT AAG GTG GAA CAA ATC TCA CAA   405

406   TTT GTA TTT CCA CCA CTC AAT TTA CTC TTC TCT TGT TTG TAT ACA   450

451   CAT ATG TAA TTC AAA GGG CTT CTC AAC ATT CAG TTT ACA TAG GAA   495

496   AGT AAG GCA TCA AAT ATC ATT TTT CGT TAA ACG TGT TAA AAA AAA   540

541   AAA AAA AAA   549
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Fig.(1):. Nucleotide and deduced amino acid sequences of cloned LeINT7 cDNA. The codon usage was optimised for Arabidopsis; start codon = ATG underlined-and stop codon = TAG (denoted by an * asterisk). The total sequence corresponds to an ORF of 243 bp encoding 77 aa.

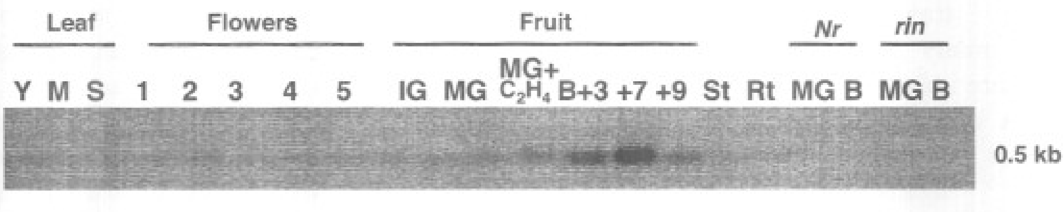

Fig.(2):. Expresion of LeINT7 mRNA in different plant organs. Y, young leaf; M, mature leaf; S, senescent leaf; Flowers1, bud; 2, open bud; 3, open flowers; 4, closed flowers; 5, senescent flowers; Fruit IG, immature green; MG, mature green; Br, breaker; St, stem; Rt, root. Forty micrograms of total RNA was extracted from tomato tissues and fruit at various ripening stages (MG, Breaker,B+3, B+7 and B+9), Full-length LeINT7 cDNA probe was used and exposure to X-ray film 24h.

Level of *LeINT7* mRNA expression in wild-type AC^{++} leaves was monitored in response to a battery of stress-inducing treatments. These treatments included: 1) SA and JA, two defence related signalling molecules; 2) ABA and NaCl, two abiotic stress-related treatments and 3) Nitric oxide (NO), a ripening- and stress-related signalling molecule. SA treatment resulted in a gradual increase in *LeINT7* transcript levels observed as early as 4 hrs post-treatment, reaching its maximum by 3d post-treatment (Fig. 3). Similar pattern of *LeINT7* transcript induction was obtained in JA-treated leaves, with stabilized maximum expression level by 1d and 2d, and diminishing by 3d post-treatment, respectively (Fig.3). Moreover, ABA-treated leaves exhibited a characteristic pattern of *LeINT7* peaking at 1d post-treatment and gradually decreasing later on. Furthermore, NaCl treatment resulted in an early accumulation of *LeINT7* transcripts (by 2 h) attaining a constitutive level of expression throughout the time course experiment (Fig. 3). It is note worthy that the spray control treatment had little or no effect on the *LeINT7* mRNA induction (Fig. 3, upper panel). Interestingly, treatment with the NO-donor SNP correlated well with a gradual increase in *LeINT7* mRNA expression, detected as early as 1h post-treatment, peaking at 8 h and decreasing by 10 h post-treatment (Fig. 4). Notably, *LeINT7* transcripts were not detected in the buffer control treatment (Fig. 4, upper panel). Taken together, these results clearly indicate that *LeINT7* might play multiple roles in fruit ripening control and various environmental stress (biotic and abiotic) signalling transduction pathways. For example, many host reactions to pathogen infection are influenced by the phytohormones C_2H_4, SA and JA (Dong, 1998). The C_2H_4-insensitive *Nr* mutant exhibits significant tolerance against virulent bacterial (*Xanthomonas campestris*pv *vesicatoria* and *Pseudomonas syringae* pv *tomato*) and fungal (*Fusarium oxysporum* f sp *lycopersici*) pathogens (Lund *et al.*, 1998). Thus, modulation of C_2H_4-depndent fruit ripening process impacts on biotic and abiotic stress signalling indicative of a certain degree of cross-talk, where different signalling pathways share one or more intermediates/components or have some common outputs. In this work, salinity and ABA treatments correlated well with the induction of *LeINT7* expression level (Fig. 3). These results are in agreement with previous reports demonstrating that salinity modulates C_2H_4-mediated signalling. For example, the non-ripening (*nor*) mutant, when grown in normal nutritional conditions, produce fruit that change colour at maturity only very slowly, whereas high salinity can partially overcome the *nor* lesion giving a fully redden and partially ripen fruit (Davies *et al.*, 1991). Moreover, it has been shown in *Arabidopsis* that osmotic stress negatively impacts on the expression level of AtETR1 resulting in increased sensitivity of the plant to C_2H_4. Thus, these results suggest that plant responses to abiotic stress are modulated by changes in the expression level of ethylene receptors (Zhao and Schaller, 2004). In this work, it was shown that NO induces the expression of *LeINT7* (Fig. 4). NO is an important signalling molecule with diverse physiological functions in plants, which was found to play a crucial role in plant growth and development, including fruit ripening pathogen resistance. In strawberry, a non-climacteric fruit, it was suggested that NO could decrease C_2H_4 output, through inhibiting 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity reducing ACC content (Zhu and Zhou, 2007). Moreover, a an inverse stoicheometric relationship between NO and C_2H_4 gases evolved from hard green and ripe fruits in avocados and strawberries, with the unripe fruit manifesting high NO and low C_2H_4- and the converse in the ripe fruit (Leshem and Pinchasov, 2000).

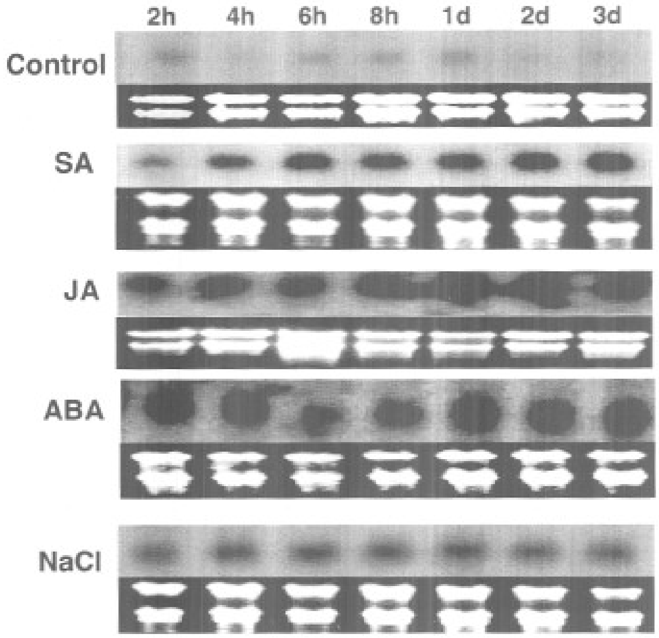

Fig.(3):. Northern gel blot analysis of endogenous LeINT7 mRNA expression in response to various stress signalling stimuli and hormonal treatments in tomato leaves. To demonstrate equal RNA loading, UV images of ethidium bromide stained total RNA are shown in the panel underneath each autoradiograph. Ten micrograms of total RNA was extracted from treated tomoto leaves at various post-treatments time points, as indicated. Full length LeINT7 cDNA probe was used for the detection of its transcript level and exposure to X-ray film at -70°C was for 6 h.

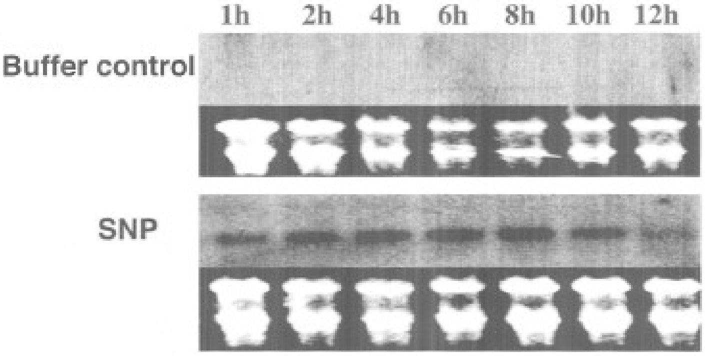

Fig.(4): Northern analysis of LeINT7 mRNA expression level in response to nitric oxide (NO). Detached leaves were incubated in a solution containing 10 mM the NO-donor SNP prepared in 10-3 M potassium phosphate buffer, pH 6.5. As a control, detached tomato leaves were incubated in the same buffer lacking SNP.

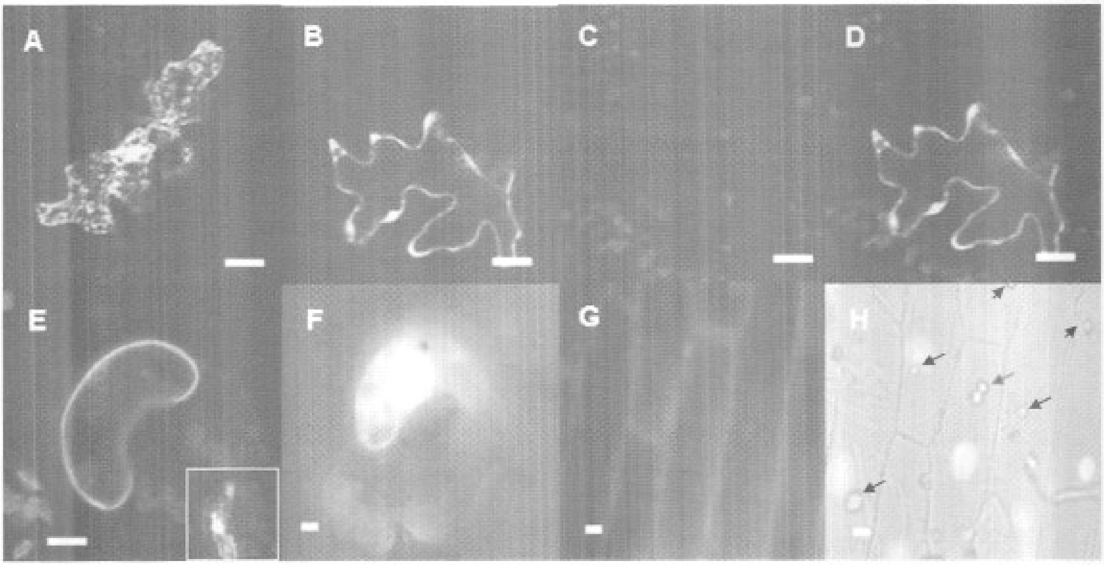

Fig.(5): Localisation of GFP-tagged LeINT7 protein in tobacco and onion epidermal and guard cells. (A-E) Confocal laser scanning microscopy images of tobacco epidermal cells expressing control Gateway.0 vector DNA containing the control 35S::GFP (A), and construct of C-terminal t® pK7FWG2agged 35S::INT7::GFP fusion without stop codon (B-D). (E) Shows the expression of C-terminal tagged LeINT7::GFP fusion contruct in a tobacco guard cell, while the inset shows tobacco guard cell expressing the 35S::GFP control DNA. Images A&B were taken with GFP channel, C with the red chlorophyl channel and D&E with red and GFP mixed channels. (F-H) Fluorescent microscopy (Leica and Nikon) of tobacco guard cell expressing the 35S::GFP control vector (F), onion epidermal cells expressing the INT7::GFP fusion protein (G&H). (G) was taken with the GFP filter and (H) was taken with the DAPI filter where the flourescent regions are cell nuclei and the bright spots (arrows) are the bombarded gold particles taken up by onion cells. Bars correspond to 20μM (A-E) and 50 μM (F-H).

In order to predict the presence of signal localisation peptides in the LeINT7 protein a group of computer-based programmes were utilised. SignalP predicted no signal peptide cleavage site in the LeINT7 protein (Prediction score: negative). Moreover, ChloroP failed to predict any chloroplast transient peptides in the LeINT7 protein (Prediction score: negative). Furthermore, search using MITOPROT and Predator resulted in a negative prediction score for the presence of any mitochondrial and plastid targeting sequences. Interestingly, protein analysis results using PSORT to predict localisation sites revealed that LeINT7 might be located on the plasma membrane (Certainty: 1.0) and the cytoplasm (Certainty: 0.65); a negative prediction for LeINT7 to be localised to the ER membrane and lumen was obtained with PSORT (Certainty: 0.0). In order to study LeINT7 localisation *in vivo* a gene construct, in which LeINT7 is C-terminally fused to GFP was generated (*35::LeINT7::GFP*). Localisation of LeINT7 expression was determined both in tobacco and onion epidermal cells, following introduction of DNA encoding GFP-tagged proteins by microprojectile bombardment. When the

35S::GFP control vector, which contained a 35S promoter and GFP with a normal stop codon, was introduced into tobacco epidermal cells by microprojectile bombardment and incubated on an 8% agar plate for 72 h. GFP was detected through the cytosol of the tobacco cell under UV (Fig. 5A). GFP-LeINT7 were similarly introduced into tobacco epidermal cells and incubated for 72h after bombardment. Expression of GFP-LeINT7 (Fig. 5B–D) was observed predominantly in the plasma membrane, compared to the location throughout the cell observed with the control GFP construct alone (Fig. 5A). Moreover, GFP-LeINT7 localisation was also found to be associated with the plasma membrane of tobacco guard cells (Fig.5E) in contrast to a localisation pattern throughout the guard cell obtained with the *35S::GFP* control vector (Inset Fig. 5E and Fig.5F). Similarly, the characteristic plasma membrane localisation was obtained with onion epidermal cells bombarded with the *35S::GFP::LeINT7* construct (Fig. 5G).

Recent studies, employing a yeast two-hybrid interaction assay, have shown that the tomato receptors (LeETR1, LeETR2 and NR) can interact with multiple LeCTRs. Moreover, *in vivo* protein localization studies with fluorescent tagged proteins indicated that NR is targeted to the ER, whereas the LeCTR proteins were found in the cytoplasm and nucleus. Authors proposed that C_2H_4 receptors recruit these LeCTR proteins to the ER membrane through direct protein–protein interaction (Zhong *et al.*, 2008). Therefore, the clear location of the LeINT7 in the plasma membrane suggests that for a NR-LeINT7 interaction to occur LeINT7 might need to be recruited from the plasma membrane to the ER, where NR is located. Based on the results presented in this work (Fig. 3 and 4), it is conceivable that the plasma membrane location of LeINT7 might be important to possibly a function in relation to biotic and abiotic stress signalling. We, therefore, envisage that future work focusing on the analyses of transgenic tomato plant mis-expressed in the LeINT7 and biomolecular fluorescence complementation studies should contribute towards underpinning the roles that LeINT7 might play in fruit ripening- and stress-related signalling.

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الملخص العربي

التعرف علي و تحديد مكان التواجد الخلوي لـ *LeINT7* : جين جديد من الطماطم له علاقة بعملية نضوج الطماطم والاستجابة لعوامل الإجهاد

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للتعرف على مكونات جينية جديدة ذات الصلة بعملية نضوج الثمار و المعتمدة على هرمون الإيثيلين فقد استفيد في السابق من عمل مسح بأسلوب Two-hybrid فى الخميرة باستخدام الـ cDNA الخاص بمستقبل الإيثيلين *NR* كطعم و الذي يزيد مستوى التعبير عنه خلال عملية النضوج. و أدى المسح الشامل لمكتبة جينية إلى التعرف على بروتين LeINT7 من خلال ارتباطه القوى و المتخصص ببروتين NR. و قد كان الهدف من هذا البحث هو التعرف و التوصيف لجين *LeINT7* المرتبط بـNR و بالتالي تحديد قدرته على التعبير الجينى تحت ظروف من الإجهاد المختلفة التي تم تجريبها, وأيضا معرفة مكان تواجده داخل الخلية وبدراسة تسلسل هذا الجين وجد أنه جين صغير ويتكون من حوالى ٢٤٣ نيكليوتيدة والذى يترجم الى بروتين LeINT7 الذي يحتوى على ٧٧ حامض أمينى. هذا و قد أوضحت الدراسة أن هذا الجين يظهر استجابة لكثير من المركبات كعوامل منظمة لتعبيره الجينى (نسخ الى mRNA) , ومن هذه العوامل حامض السالسيساليك , حامض الأبسيسيك, حامض الجاسمينك , وأكسيد النيتريك و الملوحة .كما أمكن ربط البروتين الخاص بـ LeINT7 من الطرف الكربوكسيلى مع البروتين الخاص باظهار الفلورة (GFP) و التعبير عنه بصورة مؤقتة في النبات باستخدام تكنيك قذف الجسيمات الدقيقة بهدف التعرف على مكان تواجده بالخلية , وأمكن اثبات أن هذا البروتين يوجد في أوراق الدخان و البصل كما أنه يتكون فى الغشاء البلازمى للخلية.