Asia Pacific Journal of Molecular Biology and Biotechnology, 2006
Vol. 14 (1) : 19-26

Isolation and characterization of a novel pistil predominant gene that binds weakly to the chitinase, Chi2;1 promoter of tomato.

Pick Kuen Chan[1], Hirzun Mohd Yusuf[2], Clemente Michael Wong Vui Ling[3] and Harikrishna Kulaveerasingam[2]

[1]Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia.
[2]Sime Darby Technology Centre, 2 Jalan Tandang, 45060, Petaling Jaya, Selangor Darul Ehsan, Malaysia.
[3]Biotechnology Research Institute, Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia.

Received 28 July 2005 / Accepted 10 December 2005

Abstract. A gene LN2-1-1 identified by screening a tomato pistil cDNA library has been found to encode a basic protein containing ankryin repeats. This clone was isolated by its binding ability to a specific DNA fragment in the tomato floral chitinase promoter using a yeast one hybrid system. An analysis of the temporal and spatial patterns of gene expression through northern blotting has demonstrated the highest level of expression in mature tomato pistils. *In situ* hybridization revealed that the mRNA was maximal on the upper half of the style and decreased in a gradient from the top to the lower half of the style. Hybridization was also detected on the stigma, ovules and ovary walls. The LN2-1-1 could possibly play a role in modulating the defense mechanism of tomato flowers based on its ability to bind to the chitinase, Chi 2;1 promoter.

Keywords. **Chi2;1, tomato, yeast one-hybrid system**

INTRODUCTION

Flowers play an essential role in the life cycle of flowering plants. It has become an icon in developmental studies as a vast majority of the 250, 000 angiosperm plant species have flowers with a conserved organ plant bearing sepals, petals, stamens and carpels (Espinosa-Soto, *et al.*, 2004**)**. Successful plant reproduction relies on flowering at the appropriate time followed by the progressive development of floral structures and their subsequent pollination. The range of events involved in flower development makes it an excellent model system for understanding plant development in general. Understanding the nature of the protein products of tissue-specific genes and regulatory factors that control their temporal and tissue-specific expression will provide important insights into the mechanisms of flower formation and function.

A putative potato homologue of the tomato Chi2;1 gene SK2, has also been isolated (Wemmer *et al.*, 1994) and shown to encode an endochitinase that like Chi2;1 is distinct from wound-induced chitinases. The authors demonstrated by immunocytochemistry that the protein is located to the intercellular matrix of the transmitting tissue of the style. The promoter of the potato SK2 gene has been characterized by deletion analysis, where GUS reporter constructs containing promoter fragments ranging from 1 kb to 0.23 kb conferred high levels of GUS activity in pistils of transgenic plants (Ficker *et al.,* 1997). This result demonstrated small but significant differences in the pattern of expression elsewhere in the transgenic tobacco plants compared to that reported for the full length Chi2;1 promoter (Harikrishna *et al.,* 1996). However, an ongoing experiment has demonstrated that the 435 bp fragment upstream from the start point of the Chi2;1 promoter was able to direct transmitting tissue-specific expression in the mature tomato pistils (Siti Suhaila and Harikrishna, pers. comm.). Therefore, it is likely that the regulatory elements of the Chi2;1 promoter responsible for

*Author for Correspondence.
Mailing address: P.K. Chan: Australia Research Council Centre of Excellence for Integrative Legume Research, The University of Queensland, 4072 Brisbane, Australia. Tel: +61 7 3365 4436; Fax: +61 7 3365 3556; Email: p.chan1@uq.edu.au

conferring tissue-specific expression are located within this 435 bp fragment.

In this paper, we report the isolation and characterization of a novel pistil predominant cDNA clone from a pistil cDNA library using a yeast one-hybrid screening approach. A 269 bp fragment upstream from the start point of the Chi2;1 promoter was subjected to screening. This fragment that covers the upper end of the 435 bp fragment was chosen to screen for possible *cis* acting elements.

MATERIALS AND METHODS

Plant materials and growth conditions. Tomato plants (*Lycopersicon esculentum* MILL. cv. MTII) were grown under open cultivation. The tomato flowers at anthesis were collected for dissection of petals, pistils, sepals and stamens.

Construction of a cDNA-AD fusion library. Total RNA was isolated from floral tissues as described previously by Rochester et al. (1986). Poly $(A)^+$ RNA was isolated from total RNA by using the μMACs mRNA Isolation Kit (Miltenyi Biotec, Germany). For one-hybrid screening, a cDNA-AD fusion library was constructed from poly $(A)^+$ RNA prepared from mature tomato pistils using a HybriZAP-2.1 XR (Stratagene) and a HybriZAP-2.1 XR Library Construction Kit (Stratagene). A good quality primary phage library with a titer of 3.34 x 10^6 pfu/ml was successfully constructed. All experimental procedures were performed as described in the protocol provided with the kit. For yeast one-hybrid screening, pAD-GAL4/cDNA plasmids from the lambda HybriZAP-2.1 XR phage library were excised according to the manufacturer's instructions (Stratagene).

Construction of target reporter and yeast reporter strains. The 269 bp polymerase chain reaction amplified fragment from between positions -690 to -422 of the Chi2;1 promoter, was ligated as a *Hin*dIII and *Xho*I fragment into the multicloning site (MCS) of the pLacZi expression vector (Clontech) upstream from the *lacZ* minimal promoter. The structure of the fusion construct was confirmed by sequencing. To prepare a reporter strain of yeast, the target-reporter constructs after linearization with NcoI were integrated into the *ura3* of the yeast strain YM4271 using the Matchmaker One-hybrid System protocol (Clontech) and Yeast Protocols Handbook (Clontech). Colonies that grew on the SD/-Ura plates were selected. To confirm integration of the constructed yeast reporter strains, PCR amplification was performed at the chromosomal site of integration. To determine the background expression of the constructed reporter strain, a â-galactosidase filter assay was performed as described in the Yeast Protocols Handbook (Clontech). If the lifted colony turned blue within 15 min, the background *lacZ* expression was considered to be high. However, all colonies of the strains tested only turned a weak blue after 8 hours, indicating that the background *lacZ* expression in the constructed reporter strains was low.

Yeast one-hybrid screening. For yeast one-hybrid screening, pAD-GAL4/cDNA plasmids from the lambda HybriZAP-2.1 XR phage library were excised and transformed into two yeast reporter strains containing the 269 bp Chi2;1 promoter fragment respectively according to the manufacturer's protocol (Clontech Matchmaker One-hybrid System).

β -galactosidase colony-lift filter assay. The putative positive yeast clones were grown for 3 days and assayed for β-galactosidase activity. The yeast cells were lifted onto sterile Whatman #5 filter paper, then permeabilized by immersion in liquid nitrogen for 10 s, thawed at room temperature for several minutes, and placed onto the presoaked filter paper that was prewetted with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) containing X-gal (5- bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 1 mg/mL).

Yeast protein extract. Yeast protein extracts were prepared as described previously by Arndt *et al.* (1987). The protein concentration was determined by use of a Bradford assay supplied by Bio-Rad.

Gel Mobility Shift Assay. The gel mobility shift assay was performed as described by Hadfield *et al.* (1995), except that the DNA-protein binding reaction was performed by incubating 25 ng of biotinylated DNA fragment.

Southern and Northern Blot Analyses. Total RNA was obtained from leaves, petals, pistils, sepals and stamens. The RNA samples were separated on 2% formaldehyde gels and transferred onto N-Hybond membranes (Amersham) as described by Sambrook *et al.* (1989). Filters were hybridized overnight at 42°C in 5 X SSPE, 50% deionized formamide, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml herring sperm DNA, and washed in 2 X SSC, 0.1% SDS at 42°C.

DNA extraction was performed using the CTAB method as previously described (Kidwell and Osborn, 1992). Samples (20 μg) were digested, fractionated by agarose gel electrophoresis, and blotted onto N-Hybond membranes (Amersham). Hybridization was performed as described by Montag *et al.* (1995).

In Situ *Hybridization.* *In situ* hybridization was performed as described by Jackson (1991) and Vielle-Calzada *et al.* (1999) with modifications. For synthesis of sense and antisense 11-digoxigenin-UTP labeled probes, a plasmid pCR®-Blunt II TOPO (Invitrogen) containing a 290 bp fragment (nucleotides 10-300) of the LN2-1-1 cDNA was linearized with restriction enzymes that cut within the polylinker (*Xho*I and *Bam*HI, respectively), and 1 μg was used as a template for probe

synthesis. The dissected flowers were fixed in ethanol, acetic acid and formamide in a ratio of 50:10:5 and embedded in Paraplast. Sections with thickness of 8 ì m were cut using a Leica microtome and attached onto poly-lysine coated slides. After dewaxing and hydration, slides were digested with 1 µg/ml of proteinase K for 30 min at 37°C. Following a 10 min postfixation in 4% paraformaldehyde, slides were dehydrated and immediately processed for hybridization. RNA probes were hydrolyzed as described (Jackson, 1991), and 100 ng of each labeling reaction were mixed with 40 µl of 50% formamide, added to 160 µg hybridization buffer, and used as a probe for a pair of slides. The hybridization and immunological detection were performed as according to the Vielle-Calzada *et al.* (1999).

Nuclear localization construction and transient expression of the fusion gene. The coding region of clone LN2-1-1 was prepared by polymerase chain reaction using primer set 5'-CGCGCCATGGAGGAGGATCAGGTT-3' and 5'-CGCGCCATGCCTTTTCCGAGCCTG-3'. The resulting product was inserted into the *Nco*I site of pCAMBIA1301 (CAMBIA) so as to be fused in frame to the N terminal end of GUS, downstream of the 35S promoter. The resulting recombinant DNA (1 µg) was used to coat 6 mg of 1 µm gold particles and was introduced into onion epidermal cells with a particle-delivery system (Bio-Rad PDS-1000/He). The onion epidermal cells had been preincubated on MS media supplemented with 200 µM of D-sorbitol and 200 µM D-mannitol in 24 hour light at 22°C. The initial pressure of bombardment was 1100 psi, and the traveling distance of the particles to the plant tissue was 6 cm. Histochemical staining was performed 24 hr after the bombardment as described by Sakai *et al.* (2000).

RESULTS

Identification of cDNAs encoding DNA binding proteins by using a yeast one-hybrid system. A yeast one-hybrid system was used to identify cDNAs encoding DNA binding proteins that interact with the Chi2;1 promoter region. We first constructed a parental yeast strain carrying the *lacZ* with 269 bp fragments of the Chi2;1 promoter upstream of the TATA element. The resulting yeast strains were separately transformed with a tomato pistil AD fusion library and 2.25 x 10^6 yeast transformants of the library screened. Three colonies were isolated based on their ability to induce lacZ activity and to form blue colonies on filter papers containing X-gal. The cDNA inserts of the isolated plasmids were analyzed by restriction enzyme digestion and by DNA sequencing. One of the clones designated LN2-1-1 was of 1213 bp in length with a putative open reading frame from position 747 to 1127 encoding a protein of 127 amino acids as shown in Figure 1. The predicted protein has one incomplete and two complete ankyrin repeats and a portion of a PWWP domain (Figure 1). The hydropathy plot of the predicted LN2-1-1 amino acid sequence indicates that LN2-1-1 possibly has at least 5 surface regions of globular structure with a hydrophobic N-terminal (Figure 2). The predicted protein is basic with a calculated pI of 9.25. A comparison of the amino acid sequence of this clone with the database of known sequences did not reveal any obvious homology with genes of known function. The 5' end of the cDNA clone contained a simple sequence repeat of $(AT)_9$ from positions 190 to 208.

The ankyrin repeat is a ~33 residue repeating motif that has been recognized in more than 400 proteins, including cyclin-dependent kinases, inhibitors, transcriptional regulators and toxins (Michaely and Bennett, 1992). These molecules are present in the nucleus, cytoplasm and the extracellular milieu. The number of repeats between one ankryin protein and another is highly variable. It has been well documented that the ankryin repeats are involved in mediating protein-protein interactions. The ankryin proteins do not bind selectively to a single class of protein targets. The diversity of unrelated biological roles of ankryin proteins is paralleled by the diversity of unrelated proteins with which they interact (Sedgwick and Smerdon, 1999).

The PWWP domain is a weakly conserved sequence motif found in >60 eukaryotic proteins. The PWWP motif was first identified in a gene family related to Hepatoma-derived growth factor (HDGF) (Izumoto *et al.,* 1997) and WHSC1 genes (Wolf-Hirschhorn Syndrome Candidate) (Stec *et al.,* 1998). Most of the PWWP domain proteins appear to be nuclear localized and often are DNA binding proteins that function as transcription regulators of developmental processes like WHSC1 (Stec *et al.,* 1998), BS69 (Hateboer *et al.,* 1995) and mammalian DNA methytransferase Dnmt3b (Qiu *et al.,* 2002). The PWWP domains have been hypothesized to be involved in mediating protein-protein interactions (Stec *et al*., 2000). However, Qiu *et al.* (2002) has recently shown that the PWWP domain alone can bind to DNA *in vitro*, probably through interactions with its basic surface. The functions of the PWWP domain proteins in plants have not been reported. The exact function of the PWWP has remained elusive because many proteins containing this domain have not been characterized. The PWWP domain in clone LN2-1-1 consists of KEKKKK. The four basic lysine residues, KKKK could also play a role in nuclear localization. Further characterization of the protein encoded by clone LN2-1-1 needs to be carried out to determine whether it has DNA binding specificity or exhibits universal DNA binding characteristics. Future experiments using techniques like yeast two-hybrid analysis to isolate and characterize the interacting partners with either ankyrin repeats or a PWWP domain should be carried out. These experiments could unravel the biochemical pathways in which the respective proteins are involved, and the precise function of the protein encoded by LN2-1-1.

```
1    GGATCCTCTGCTAGCAGACAATTCGGCACGAGGTTCTCTTCTTCCTTCTTCCTCCCAATT 60
61   CCTTCAAAACCCAAGCCTAATCAAACAAGAAAATCCCTTTATCAATACTTATGCAACCTC 120
121  CAACCATATTCCAAATTCATCAAGGATTCATATAATAGCCCTTTTTTTCCCCGTAAAAAA 180
181  ACAGCTATTATATATATATATATATATTGTTTGCGTGGTTTTTTGCATTGATAATGATAA 240
241  TCTCTGCTTTCCTTCGTTTCACTTCATCTTGTGTGTTCAATAAGCTTACAAATCTTCGTG 300
301  TTTTTGGTCCTGGATTGAACCCTTTTGGTCCTTACAACTTGGCTAATCATTCCTCTCGCT 360
361  AAAGCCCTAATTTTTTTCTTGGTTGGTTGTTTGCCTCTGGATCATGTAAAATTGAAGGTT 420
421  GTTTCAGAAGATTATCACTTGTTTTGGTTCTTGTCTATTGAAGACTTTTCTGGGTTTCTC 480
481  TCATAGTCGGAAGACAGATCAATCTTGTTGGTCAAAGTTATTAGTTTTATGTAAAAGGTC 540
541  TTGTTTTGGTGTATTGAGATTCAGTTACTGATATACAAGTAGAGTCTTTAGACTTGTTGA 600
601  TTAGGTTTTCGAAATTTCTCTTCCGGACACTGGTGAAAGAAGATTCTACAAGAAAGAATA 660
661  GAATTGGATTGAGTATTTGTTTCAATTTGTGGCAATAAGGATTGGATTTTGGAGCTTTGC 720
721  TTATTAGGAAAGCGGAGAAGAGGAAAATGGAGGAGGATGAGGTTGGTTCGACTCGACAAT 780
                                M  E  E  D  E  V  G  S  T  R  Q

781  CGAGAAGAATGCGTATAACTGCAGGTGATACGGATGATCGCGGTTGGACTCCACTTCACA 840
     S  R  R  M  R  I  T  A  G  D  T  D  D  R  G  W  T  P  L  H

841  TTGTTGCTCGGAAAGGTGACCTGAAACAGGTTAGAAGGCTTCTTAATGAAGGTATGGATG 900
     I  V  A  R  K  G  D  L  K  Q  V  R  R  L  L  N  E  G  M  D

901  CAAATGTGATGGCAGGAGGCCCAAAATCATTTGGTATGACCCCACTTCATCTTGCTGCTA 960
     A  N  V  M  A  G  G  P  K  S  F  G  M  T  P  L  H  L  A  A

961  AGGGAGGTCACGTGAGAGTTATGGATGAATTACTTGAGAGGGGTGCTGATATCGATGCTC 1020
     K  G  G  H  V  R  V  M  D  E  L  L  E  R  G  A  D  I  D  A

1021 GAGCCAAGGGTGCATGTGCATGGACTCCTCTCCATCATGCTGCGAAAGAGAAAAAGAAGA 1080
     R  A  K  G  A  C  A  W  T  P  L  H  H  A  A  K  E  K  K  K

1081 AAGCGATGGTGTCAGGGTGCGTAAGTCTAAGTTGCAGGGACCAATGGTAGACCTATCTNG 1140
     K  A  M  V  S  G  C  V  S  L  S  C  R  D  Q  W  *

1141 GTAGGGTCAGGCGAAGGATTCATTAATGANTCCNGNAAACNAAGTAGGTAGTCGAAAGGC 1200
1201 ATCAGAAAAAGAA
```

Figure 1. Nucleotide and deduced amino acids sequence of clone LN2-1-1. The '*' indicates the stop codon of the ORF (represented by the amino acid sequences). Solid lines with arrow, ankryin repeats; dashed line, PWWP domain.

Spatial Expression Pattern of LN2-1-1. The spatial expression pattern of LN2-1-1 was determined by northern blot hybridization (Figure 3). The strongest expression of the LN2-1-1 gene was detected in pistils from flowers at anthesis. Weak hybridization signals were visible in the lane of petals and weaker signals in sepals from flowers at the same stage. These results indicate that the LN2-1-1 gene is predominantly expressed in pistils at anthesis. As the expression pattern of LN2-1-1 gene is similar to Chi2;1 (Harikrishna *et al.,* 1996), this provides further evidence that the LN2-1-1 gene encodes for a protein that is likely to interact with the Chi2;1 promoter.

The copy number of LN2-1-1 gene in the tomato genome was estimated by Southern blot analysis (Figure 4). Genomic DNA from tomato was restriction digested with *Eco*RV, *Hind*III and *Xba*I to reveal simple digestion patterns. Genomic analysis suggests that a single gene may encode LN2-1-1.

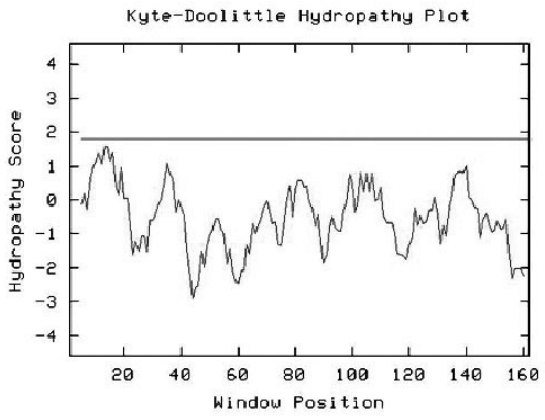

Figure 2. Hydropathy plot (Kyte-Doolittle, 1982) of LN2-1-1 with a calculated pI of i9.25. The line indicates possible transmembrane regions.

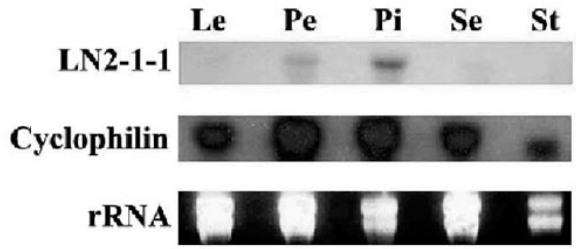

Figure 3. Northern analysis of the LN2-1-1 transcripts. Spatial expression pattern of LN2-1-1 gene in a variety of tomato organs. Each lane was loaded with 20 µg of total RNA from leaves (Le), petals (Pe), pistil (Pi) and sepals (Se). As a control for RNA loading, the filter was stripped and reprobed with a tomato cyclophilin cDNA fragment that showed expression in all organs. rRNA blotted on the membrane was visualized by staining with ethidium bromide as an additional control for loading.

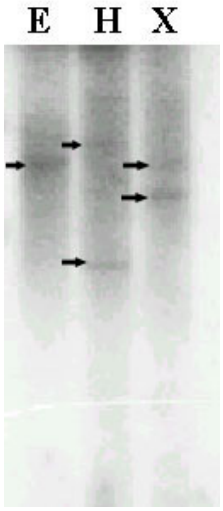

Figure 4. Southern analysis of LN2-1-1. Genomic DNA was digested with *Eco*RV (E), *Hind*III (H) and *Xba*I (X). A full length LN2-1-1 cDNA was used as probe.

Analysis and characterization of the DNA Binding Affinity of the LN2-1-1 Protein. In order to ensure that the *in vivo* binding activity of LN2-1-1 determined by yeast one-hybrid analysis was not due to a spurious interaction another independent method was employed. The results obtained from this test could provide convincing evidence to support whether the isolated clone encodes a DNA binding protein. Therefore an *in vitro* binding gel shift DNA binding assay was performed to verify the results obtained from yeast one-hybrid screening. Native yeast protein extracts of LN2-1-1 clone was prepared for an *in vitro* binding assay with the 269 bp fragment of the Chi2;1 promoter. The yeast protein extracts of LN2-1-1 interacted with the 269 bp fragment in the form of a shifted DNA band (Figure 5). However, when unlabeled fragment of 269 bp was added as a cold competitor, the shifted band remained. This shows that the binding of the LN2-1-1 protein was non-specific. The positively charged PWWP domain might interact with negatively charged molecules such as DNA (Qiu *et al.,* 2002). Therefore, these four lysine residues might contribute a universal DNA binding property to clone LN2-1-1.

Nuclear localization of LN2-1-1. The deduced LN2-1-1 protein contains the KKKK sequence. These basic lysine (K) residues could possibly be nuclear localization signal (Raikhel, 1992). A score of 8% for nuclear targeting was obtained the predicted protein using analysis tool for protein subcellular localization prediction, WoLF PSORT at http://wolfpsort.seq.cbrc.jp/. A prediction confidence of 87.6% for nuclear targeting was obtained using a another software, pTARGET program at http://bioinformatics.albany.edu/~ptarget (Guda and Subramaniam, 2005). However, the pTARGET method is primarily used to predict the subcellular localization of eukaryotic and non- plant sequences. We performed an *in vivo* targeting experiment using a LN2-1-1 GUS fusion gene to determine whether LN2-1-1 has the potential for nuclear targeting. We generated the LN2-1-1-GUS fusion within the pCAMBIA1301 vector (CAMBIA). Both recombinant DNA constructs encoding a LN2-1-1-GUS fusion and a control GUS protein alone were introduced into onion epidermis cells by particle bombardment (Sakai *et al.,* 2000). Histochemical analysis revealed GUS activity in the cell indicating that the LN2-1-1-GUS fusion protein remained in the cytoplasm. The control construct showed GUS protein localization in the cytoplasm (data not shown). These results suggested that the KKKK sequence in the predicted protein of LN2-1-1 is not likely to be involved in nuclear localization but may contribute a universal binding property.

Tissue specific expression of LN2-1-1 in specialized tissues. Northern blot analysis showed that the expression of LN2-1-1 was pistil predominant with lower expression in the petals. The expression pattern of LN2-1-1 was similar to Chi2;1 (Harikrishna et al., 1996). To confirm the tissue specific pattern of gene expression, tissue specific localization of LN2-1-1 mRNA transcripts, were determined by *in situ* hybridization to tissue layers of the transmitting style on a cross section taken through the stigma. Figure 5a shows the result of hybridization of antisense LN2-1-1 mRNA to a longitudinal tomato pistil section. Hybridization is maximal on the upper half of the style and decreases in a gradient from top to the lower half of the style. At the top of the style, hybridization was localized to the stigma. Hybridization of antisense LN2-1-1 riboprobe to a serial cross-section of the style is shown in Figure 6. Figure 6a-d demonstrated the localization of LN2-1-1 to the stigma and the transmitting

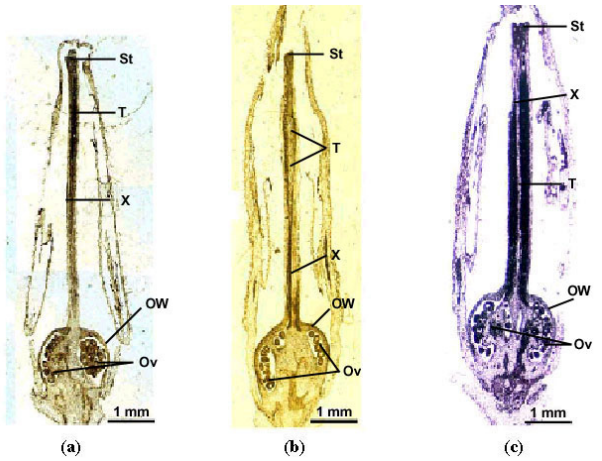

Figure 5. *In situ* hybridization analysis of pistil longitudinal-sections. (a) A longitudinal section of a mature tomato pistil was hybridized with probe LN2-1-1. Strong hybridization is visible in the transmitting tract of the style. (b) A longitudinal section of a mature tomato pistil was hybridized with sense probe of LN2-1-1. The xylem vessel members (X) appear black in this bright field micrograph due to their refractile walls. Previous experiments also showed clearly visible dark xylem in both sense and antisense hybridizations (Gasser et al., 1989). (c) A longitudinal section of a mature tomato pistil stained with periodic acid and napthol blue black to reveal cellular details. St, stigma; T, transmitting tissue; X, xylem; OW, ovary wall; Ov, ovules. The scale bar is 1mm.

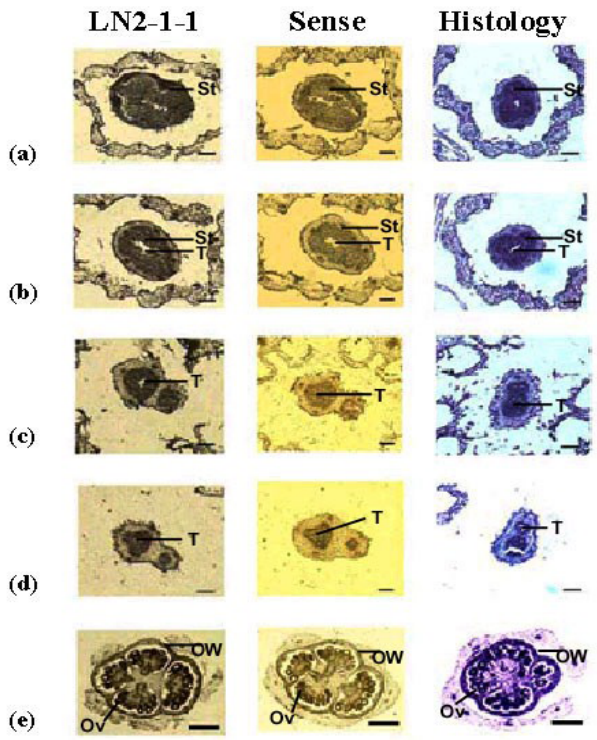

Figure 6. *In situ* hybridization analysis of mature tomato pistil cross-sections. (a) A cross-section taken through the stigma. (b) Transverse section of the upper region of the style. (c) Transverse section of the middle region of the style. (d) Transverse section of the lower region of the style. (e) Transverse section of the lower region of the pistil with ovary. St, stigma; T, transmitting tissue; OW, ovary wall; Ov, ovules. The scale bar is 1mm.

tract. A cross section of the ovary (Figure 6e) revealed that antisense LN2-1-1 hybridized to the ovary walls and ovules.

DISCUSSION

We have successfully identified a novel tomato gene using the yeast one-hybrid screening system. The LN2-1-1 protein was demonstrated to bind to a 269 bp region of the Chi2;1 promoter through mobility shift assays. This result suggests that the protein encoded by LN2-1-1 might interact with the Chi2;1 promoter. This interaction could occur in the transmitting tract of mature tomato pistils based on the results observed from *in situ* hybridized tissue sections (Figure 5, 6). However, LN2-1-1 does not have any significant sequence homology to any known proteins. The predicted protein of 127 amino acids has one incomplete and two complete ankyrin repeats and a portion of a PWWP domain.

Previously, Harikrishna et al (1996) demonstrated that Chi2;1 is predominantly expressed in mature pistils. We have demonstrated that the LN2-1-1 is predominantly expressed in mature pistils (Figure 3) and like Chi2;1 LN2-1-1 transcripts were detected at low levels in petals and sepals from flowers at the same stage. As the expression pattern of LN2-1-1 gene is similar to that of Chi2;1, the tissue specific localization of LN2-1-1 transcripts was further determined via *in situ* hybridizations. LN2-1-1 mRNA localized at maximal levels on the upper half of the style and decreased in a gradient from top to the lower half of the style. LN2-1-1 transcripts were also detected on the stigma, ovary walls and ovules. While Gasser et al. (1989) showed that the localization of Chi2;1 to the transmitting tissue of the style, ovary walls and ovules, the great similarity between the pattern of mRNA distribution of Chi2;1 and LN2-1-1 suggests that LN2-1-1 might interact with the Chi2;1 promoter region and thereby influence Chi2;1 gene expression to a certain extent.

Although the exact function of LN2-1-1 protein in tomato style has not yet been determined their mRNA localization in mature tomato style indicate that they might play a critical role in mediating plant defense based on their ability to bind to the Chi2;1 promoter. The transmitting tissue of the style represents an open, nutritionally rich channel with high humidity, which constitutes an ideal environment for fungal

growth (Gasser, 1991). This suggests that LN2-1-1 may be expressed in the style to mediate the defense mechanisms in preventing pathogen infection of the reproductive structure. The LN2-1-1 protein might be produced in response to pathogen infection. However, it is possible that the defense-related genes might play other alternative roles in the plants. For example Ori *et al.* (1990), have prostulated that a stylar â-(1,3)-glucanase gene may be involved in facilitating pollen tube extension. The transcript of LN2-1-1 is expressed primarily in the upper half of the style suggested that other transcription factors such as LeCBP (Chan *et al.*, 2004) might be involved in maintaining this high expression of defense genes throughout the style.

Further characterization such as transcription activation and the introduction of LN2-1-1 into plants through transformation could possibly provide more information on the function of this gene. The use of some other techniques such as yeast two-hybrid analysis could facilitate the isolation and characterization of interacting partners. These experiments should allow the unraveling of biochemical pathways in which the respective proteins are involved, as this gene does not have significant homology to known proteins in the databases, thereby increasing our understanding about their precise function.

ACKNOWLEDGEMENTS

This work was supported by Top-Down grant for molecular biology (Grant no: 29-02-03-0001) from the National Biotechnology Directorate, Ministry of Science Malaysia. We thank Drs Ho Chai Ling, Jennifer Ann Harikrishna, and Peter Gresshoff for critical comments and helpful suggestions. We gratefully acknowledge Dr Vila (Malaysia Agriculture and Development Institute), Dr Meilina Ong Abdullah (Malaysia Palm Oil Board, [MPOB]), Dr. Sharifah Shahrul Rabiah Syed Alwee (MPOB), Zaidah Rahmat (MPOB) and Dr Tan Siang Hee (Universiti Putra Malaysia).

REFERENCE

Arndt, K.T., Styles, C. and Fink, G.R. (1987) Multiple global regulators control *HIS4* transcription in yeast. *Science* 237:874-880.

Chan, P.K., Yusuf, H.M., Ling, C.M.V.L., and Kulaveerasingam, H. (2004) The identification and characterization of a novel transcription factor LeCBP, a potential regulator of the tomato stylar Chi2;1 promoter. *Plant Science* 167:1291-1300.

Espinosa-Soto, C., Padilla-Longoria, P. and Alvarez-Buylla, E.R. (2004) A gene regulatory network model for cell-fate determination during *Arabidopsis thaliana* flower development that is robust and recovers experimental gene expression profiles. *The Plant Cell 16:2923-2939.*

Ficker, M., Wemmer, T. and Thompson, R.D. (1997) A promoter directing high level expression in pistils of transgenic plants. *Plant Mol Biol* 35:425-431.

Gasser, C.S. (1991) Molecular studies on the differentiation of floral organs. Ann Rev *Plant Physiol* 42:621-649.

Gasser, C.S., Budelier, K.A., Smith, A.G. Shah, D.M., Fraley, R.T. (1989) Isolation of tissue-specific cDNAs from tomato pistils. *Plant Cell* 1:15-24.

Guda, C. and Subramaniam, S. (2005) pTARGET: a new method for predicting protein subcellular localization in eukaryotes. *Bioinformatics* 21:3963-3969.

Hadfield, C., Mount, R.C. and Cashmore, A.M. (1995) Protein binding interactions at the STB locus of the yeast 2ìm plasmid. *Nucleic Acids Res* 23:995-1002.

Harikrishna, K., Jampates-Beale, R., Milligan, S.B., Gasser, C.S. (1996) An endochitinase gene expressed at high levels in the stylar transmitting tissue of tomatoes. *Plant Mol Biol.* 30:899-911.

Hateboer, G., Gennissen, A., Ramos, Y.F.M., Kerkhoven, R.M., Sonntag-Buck, V., Stunnenberg, H.G. and Brenards, R. (1995) BS 69, a novel adenovirus E1A-associated protein that inhibits E1A transactivation. *EMBO J.* 14:3159-3169.

Izumoto, Y., Kuroda, T., Harada, H., Kishimoto, T. and Nakamura, H. (1997) Hepatoma-derived growth factor belongs to a gene family in mice showing significant homology in the amino terminus. *Biochem. Biophys. Res. Commun.* 238:26-32.

Jackson, D.P. (1991) *In situ* hybridization in plants. *In Molecular plants pathology: A practical approach* (ed. D.J. Bowles, S.J. Gurr, and M. McPhereson), pp. 163-181. Oxford University Press, Oxford, UK.

Kidwell, K.K. and Osborn, T.C. (1992) Simple plant DNA isolation procedures. *In Plant Genomes: Methods for Genetic and Physical Mapping* (ed. J. S. Beckmann and T. C. Osborn), pp. 1-13. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Michealy, P. and Bennett, V. (1992) The ANK repeat: a ubiquitous motif involved on macromolecular recognition. *Trends Cell Biol.* 2:127-129.

Montag, K., Salamini, F and Thompson, R.D. (1995) ZEMa, a member of a novel group of MADS box genes, is alternatively spliced in maize endosperm. *Nucleic Acids Res* 23:2168-2177.

Ori, N., Sessa, G., Lotan, T., Himmelhoch, S. and Fluhr, R. (1990) A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superclass. *EMBO J* 9:3429-3436.

Qiu, C., Sawada, K., Zhang, X. and Cheng, X. (2002) The PWWP domain of mammalian DNA methytransferase Dnmt3tb defines a new family of DNA- binding folds. *Nat. Struct. Biol.* 9:217-224.

Raikhel, N. (1992) Nuclear targeting in plants. *Plant Physiol.* 100:1627-1632.

Rochester, D.E., Winter, J.A. and Shah, D.M. (1986) The structure and expression of maize genes encoding the major heat shock protein, hsp70. *EMBO J* 5:451-458.

Sakai, H., Aoyama, T. and Oka, A. (2000) *Arabidopsis* ARR1 And ARR2 response regulators operate as transcriptional activators. *Plant J* 24:703-711.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning, A Laboratory Manual. 2nd ed. New Yo`development and homologous to a *Drosophila* dysmorphy gene maps in the Wolf- Hirschhorn syndrome critical region and is fused to IgH in t(4;14) multiple myeloma. *Hum. Mol. Genet.* 7:1071-1082.

Vielle-Calzada, J.-P., Thomas, J., Spillane, C., Coluccio, A., Hoeppner, M.A. and Grossniklaus, U. (1999) Maintenance of genomic imprinting at the *Arabidopsis medea* locus requires zygotic DDM1 activity. *Genes & Dev.* 13:2971-2982.

Wemmer, T., Kaufmann, H, Kirch, H.H. Schneider K., Lottspeich, F., Thompson, R.D. (1994) The most abundant soluble basic protein of the stylar transmitting tract in potato (*Solanum tuberosum* L.) is an endochitinase. *Planta* 194:264-273.