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Changes in the expression of *CrFTA*, the *Catharanthus roseus* farnesyltransferase α-subunit gene, in response to a *Candidatus* Liberibacter asiaticus infection

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Abstract: The farnesyltransferase α-subunit (FTA) may be involved in the regulation of defence responses against pathogens in plants. In this study, this gene was amplified from *Catharanthus roseus* (*CrFTA* gene). The cDNA was found to be 1 403 bp long, and encodes a putative protein of 331 amino acids that contains a conserved PPTA motif. The phylogenetic analysis showed that the sequence of *CrFTA* is the most similar to that from *Coffea canephora*. The qRT-PCR assays indicated that *CrFTA* is expressed in the leaves, stems, and roots. During a *Candidatus* Liberibacter asiaticus (*Ca*. L. asiaticus) infection, the *CrFTA* expression levels significantly increased and reached 18-fold that measured in the control group, after which its expression decreased gradually from 22 days after top-grafting (DAT) to the end of the experiment. Spray application of Manumycin A (ManuA), a specific inhibitor of farnesyltransferase, on the leaves of *C. roseus* plants caused a significant decrease in the *CrFTA* expression and a significant increase in the *Ca*. L. asiaticus positivity percentage after top-grafting with the *Ca*. L. asiaticus-infected shoots compared with the groups not treated with ManuA. Furthermore, ABA had no significant effect on the relative expression of *CrFTA* and the number of *Ca*. L. asiaticus-positive plants. These results suggest that *CrFTA* most likely plays a role in mediating the tolerance to a *Ca*. L. asiaticus infection in *C. roseus*.

Keywords: *Ca*. L. asiaticus; gene expression; Madagascar periwinkle; Manumycin A

Huanglongbing (HLB), also known as citrus greening disease, is widely distributed in most citrus-planting countries worldwide (Deng et al. 2012). The disease is mainly associated with *Candidatus* Liberibacter asiaticus (*Ca*. L. asiaticus) in Asian countries, Brazil, and the USA (Florida) (Islam et al. 2012). Currently, *Ca*. L. asiaticus has become the most widespread and destructive pathogen of citrus in the world (Wang & Trivedi 2013). Citrus plants have a long growth period, and studying HLB in citrus *in vivo* is time consuming (Ding et al. 2015). Therefore, performing disease screening in an experimental host plant would be very convenient. *Ca*. L. asiaticus can be successfully reciprocally transmitted from the citrus to the Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don (Apocynaceae) by grafting or through dodder, and it multiplies rapidly in the phloem of infected *C. roseus* plants (Zhang et al. 2010).

Previous studies have shown that the phytohormone abscisic acid (ABA) is important for mediating abiotic stress responses, and has multifaceted functions in a plant's immunity (Cao et al. 2011). In plants, farnesyltransferase is involved in the ABA signalling pathway (Cutler et al. 1996). The farnesyltransferase protein consists of α and β subunits, and its catalytic site is formed from an interfacial cleft at the contact site between the two subunits (Ying et al. 1994). The α-subunit is responsible for the substrate binding and

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the transfer reaction, while the β-subunit is crucial for the substrate specificity. The farnesyltransferase α-subunit (FTA) also plays an important role in the regulation of the ABA concentration in *Arabidopsis* (Running et al. 2004; Wang et al. 2009), and deletion of the *FTA* coding gene results in various developmental problems including enlarged meristems and delayed growth (Running et al. 2004).

In the present study, the *CrFTA* gene was cloned and the pattern of gene transcription in response to a *Ca.* L. asiaticus infection and the *Ca.* L. asiaticus-positivity percentage in *C. roseus* after top-grafting with *Ca.* L. asiaticus were analysed.

MATERIAL AND METHODS

Plants and treatments. A total of 180 *Ca.* L. asiaticus-free *C. roseus* plants were grown in individual pots and divided into six groups, groups I–IV and the control (CK). After eight weeks, the plants in groups I–IV were top-grafted with yellow shoots of *C. roseus* plants that were confirmed to be positive for a *Ca.* L. asiaticus infection by a PCR (polymerase chain reaction) test (Zhang et al. 2010; Li et al. 2018), and the plants in the CK were top-grafted with scions from healthy *C. roseus* plants. After grafting, all the plants were kept in a humid chamber to allow the grafts to survive and were thereafter grown in an insect-proof screen house.

A specific inhibitor of farnesyltransferase, Manumycin A (ManuA; BioVision Inc., Milpitas CA, USA), and abscisic acid (ABA; BioVision) were individually dissolved in ethanol at a concentration of 100 mM. The *C. roseus* plants were foliar-sprayed with the ABA solution in group II, the ManuA solution in group III, and the ManuA + ABA solution in group IV every two days after top-grafting (DAT) as described by Jiang et al. (2010). Similarly, the plants were foliar sprayed with ethanol in group I and the CK.

Sampling. Three healthy *C. roseus* plants were randomly chosen in group CK for cloning the *CrFTA* cDNA and for determining the *CrFTA* expression pattern in the different organs prior to the foliar application of ethanol. The leaves, main stems, and roots were cut into small pieces using sterilised scissors and frozen in liquid nitrogen. One gram of each tissue (leaves, main stems, and roots) was sampled for the RNA extraction and analysing the expression levels of *CrFTA* in the different tissues of the healthy *C. roseus* plant. To analyse the expression levels of *CrFTA* in the leaves in the different groups, three *Ca.* L. asiaticus-positive plants were selected randomly from groups I–IV, and 1.0 g of leaf tissue was sampled from each for the RNA extraction at the different DAT. The total RNA was extracted from the samples using a Trizol Reagent (Invitrogen, CA, USA). The genomic DNA was extracted from 0.1 g of the leaf tissue sampled from the same plants at the same time using the method of Li et al. (2019).

Cloning the full-length *CrFTA* cDNA. A partial *CrFTA* cDNA fragment was amplified from the *C. roseus* leaf RNA by PCR using the primers CrF and CrR (Table 1). The amplification reactions were performed using the conditions at 94 °C for 5 min, then 30 cycles of 94 °C for 1 min, 54 °C for 45 s and 72 °C for 1 min, and then a final extension period at 72 °C for 10 min. 3'RACE and 5'RACE were performed with the 3'-Full RACE Core Set with PrimeScript™ RTase (Takara, Japan) and the 5'-Full RACE Kit with TAP (Takara), respectively. The sequence contigs were assembled with the SeqMan program (Lasergene, USA). A phylogenetic tree was constructed using the neighbour-joining method (Tamura et al. 2011).

Quantification of *CrFTA* mRNA levels in the different plant tissues. Assays were performed to analyse the expression pattern of *CrFTA* in the different tissues taken from the healthy *C. roseus* plants. The primers used are shown in Table 1. The 18S *rDNA* gene was used as the internal control for the gene expression. The qRT-PCR analyses were performed using a QuantiTect SYBR green PCR kit (Qiagen, Hilden, Germany) and the amplification conditions were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. Highly purified water produced by the Milli-Q® CLX 7000 Connected High-Throughput Water Purification System and total RNA extracted from the leaf tissue of the *C. roseus* plants infected with *Ca.* L. asiaticus were the negative and positive controls, respectively. The expression levels of *CrFTA* were calculated using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001). For each tissue sample, there were three biological replicates and the qRT-PCR assays were performed in triplicate.

The expression profiles of *CrFTA* in response to *Ca.* L. asiaticus infection. To determine the expression profile of *CrFTA* in the leaves during the *Ca.* L. asiaticus infection, qRT-PCR assays were performed as above using the RNA extracted from 1 g of the leaves at the different DAT for the plants in groups I–IV and CK. Each sample consisted of three biological replicates, and the qRT-PCR assays were performed three times for each replicate.

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Table 1. Primers used for the PCR in this study

Primers	Primer sequence (5'→3')	Targeting sequence	Reference
Cloning *CrFTA* sequence			
CrF	TTCTCTCATGGCCTCGATGC	the partial cDNA of *CrFTA*	this study
CrR	TTCTCTCATGGCCTCGATGC		
RACE			
FTA-5R	CTTGGCTGGGACGTAAACCA	5'-flanking sequence of *CrFTA*	this study
FTA -5NR	TTCTCTCATGGCCTCGATGC		
FTA-3F	GCGAATACTTGAGGCCCTCA	3'-flanking sequence of *CrFTA*	this study
FTA-3NF	TATGGCATCACAGGCGATGG		
qRT-PCR analysis			
FTA-F	TGGAGGTATCTTCGAGGCCT	*CrFTA*	this study
FTA-R	CTTGGCTGGGACGTAAACCA		
18S-F	GACTACGTCCCTGCCCTTTG	18S rDNA of periwinkle	Li et al. (2018)
18S-R	AACACTTCACCGGACCATTCA		
Detection of *Ca.* L. asiaticus			
OI1	GCGCGTATGCAATACGAGCGGCA	16S rDNA of *Ca.* L. asiaticus	Zhang et al. (2010)
OI2c	GCCTCGCGACTTCGCAACCCAT		

Detection of *Ca.* L. asiaticus in *C. roseus* plants by PCR. To evaluate the presence of *Ca.* L. asiaticus in the *C. roseus* plants after top-grafting in each group, PCR amplifications were performed using the DNA extracted from the leaf tissues of the experimentally infected plants according to the method of Zhang et al. (2010). One gram of the leaf tissue samples was taken from each plant, and the PCR amplification

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cctccggttcattcatgcaatctcaactcccctcattaacttactaattgcgctactggtctagaccatgcacgtttcatatgcctagga  90
tagcagcttcagcgtcattttactagtacactattctgctacaatttaactcgaccttacaaaggtcatttgaacctacaccgtagagta  180
ctttcaatggaccctgacgagttgaagcaaggtaaaagaatcccgctgagcaaacggcctgaatggtctgacgtgactcctgtcccccaa  270
      M  D  P  D  E  L  K  Q  G  K  R  I  P  L  S  K  R  P  E  W  S  D  V  T  P  V  P  Q  28
aatgatggcccaaagcctgttgttcctatctcctacagcgatgagttctctgaaaccatgaactacttcagagccatctactttgctgat  360
 N  D  G  P  K  P  V  V  P  I  S  Y  S  D  E  F  S  E  T  M  N  Y  F  R  A  I  Y  F  A  D  58
gagcgctcctcgcgagctcttcaactcactgccgaagccattaaacacaatcctggcaattacacagtatggcagtttaggcggcgaata  450
 E  R  S  S  R  A  L  Q  L  T  A  E  A  I  K  H  N  P  G  N  Y  T  V  W  Q  F  R  R  R  I  88
cttgaggccctcaatgctaacttgcaggaggaattggaatacttgggcagcattgctgaaggcaacaccaagaactatcagatatggcat  540
 L  E  A  L  N  A  N  L  Q  E  E  L  E  Y  L  G  S  I  A  E  G  N  T  K  N  Y  Q  I  W  H  118
cacaggcgatgggttgccgagaaattaggaagtgatgctagaagtaaggagctcgagttcaccaaaaaaatcttcatggatgcaaaaaac  630
 H  R  R  W  V  A  E  K  L  G  S  D  A  R  S  K [E  L  E  F  T  K  K  I  F  M  D  A  K  N] 148
tatcatgcctggtctcatagacagtgggttcttcaagcacttggtgggtgggaagatgaacttgcgtattgtcagcaactccttgaagag  720
[Y  H  A  W  S  H  R  Q  W  V  L  Q] A  L  G  G  W  E  D  E  L  A  Y  C  Q  Q  L  L  E  E  178
gacatttttaacaattctgcttggaatcagagatatttgttctaacaagatctcctctgcatggaggcatcgaggccatgagagaatct  810
 D  I  F  N  N  S  A  W  N  Q  R  Y  F  V  L  T  R  S  P  L  H  G  G  I  E  A  M  R  E  S  208
gaagtatcctatgcagttaacgcgattatatccgatccaggaaatgagagtccatggaggtatcttcgaggcctctatggcaaagacact  900
 E  V  S  Y  A  V  N  A  I  I  S  D  P  G  N  E  S  P  W  R  Y  L  R  G  L  Y  G  K  D  T  238
caatctcttagtaaagatcctcaagtggtttcagtttgcttagaggttttagcttccaagagcaactttgtccatgctctcaacatgctt  990
 Q  S  L  S  K  D  P  Q  V  V  S  V  C  L  E  V  L  A  S  K  S  N  F  V  H  A  L  N  M  L  268
ttggatcttcttttccatggtttacgtcccagccaagagttgagagatgctgtgagcgcgctaaatcctgactcaggtacgtcagattca  1080
 L  D  L  L  F  H  G  L  R  P  S  Q  E  L  R  D  A  V  S  A  L  N  P  D  S  G  T  S  D  S  298
gatttggcgataacaatctgcattatcttggaacgcgatgaccccataagacaaaactactggaagtggcggagaaacagagtgaacaat  1170
 D  L  A  I  T  I  C  I  I  L  E  R  D  D  P  I  R  Q  N  Y  W  K  W  R  R  N  R  V  N  N  328
ggagcatcttaactgtgtgactacttttcgataaccactaatcccatatccaatagatcatactaactgctacatagcattatctgcatg  1260
 G  A  S  *                                                                                 331
cacgatatactaagcaatctagtcttcactatggtttagacaataaacagttgactattttaatctgtacatcgatcataaactgtcata  1350
tttgcaaaatgatgcagcagaagctcgaatctgaaaaaaaaaaaaaaaaaaaa  1403
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Figure 1. The nucleotide and deduced amino acid sequences of the farnesyltransferase α-subunit gene, *CrFTA*, from *Catharanthus roseus* (GenBank accession No. MH461104)

The asterisk indicates the stop codon; the polyadenylation signal (AATAAA) is shown in italics; the conserved PPTA motif is boxed

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of *Ca*. L. asiaticus was performed in triplicate on each sample. Milli-Q® water and DNA including the genome of *Ca*. L. asiaticus were used as the negative and positive controls, respectively. The *Ca*. L. asiaticus-positive percentage was calculated using the following formula: *Ca*. L. asiaticus-positive percentage = the number of *Ca*. L. asiaticus-positive plants/ the total number of experimental plants × 100%.

Statistical analysis. The data were analysed using a one-way analysis of variance (ANOVA) followed by Dunnett's test, and the statistical significance was defined as $P < 0.05$. The results are expressed as the mean ± SD.

RESULTS

cDNA cloning and sequence analysis of *CrFTA*. The full-length *CrFTA* cDNA is 1 403 bp in length with a 996-bp ORF, 186-bp 5'-untranslated region, and a 221-bp 3'-untranslated region (Figure 1). The CrFTA protein is predicted to contain a conserved PPTA motif. The Neighbour-Joining method was used to perform a phylogenetic analysis of the CrFTA with FTA sequences from 27 other species of plants (Figure 2). The resulting phylogenetic tree showed that the CrFTA clusters with a predicted FTA protein were from *Coffea canephora*.

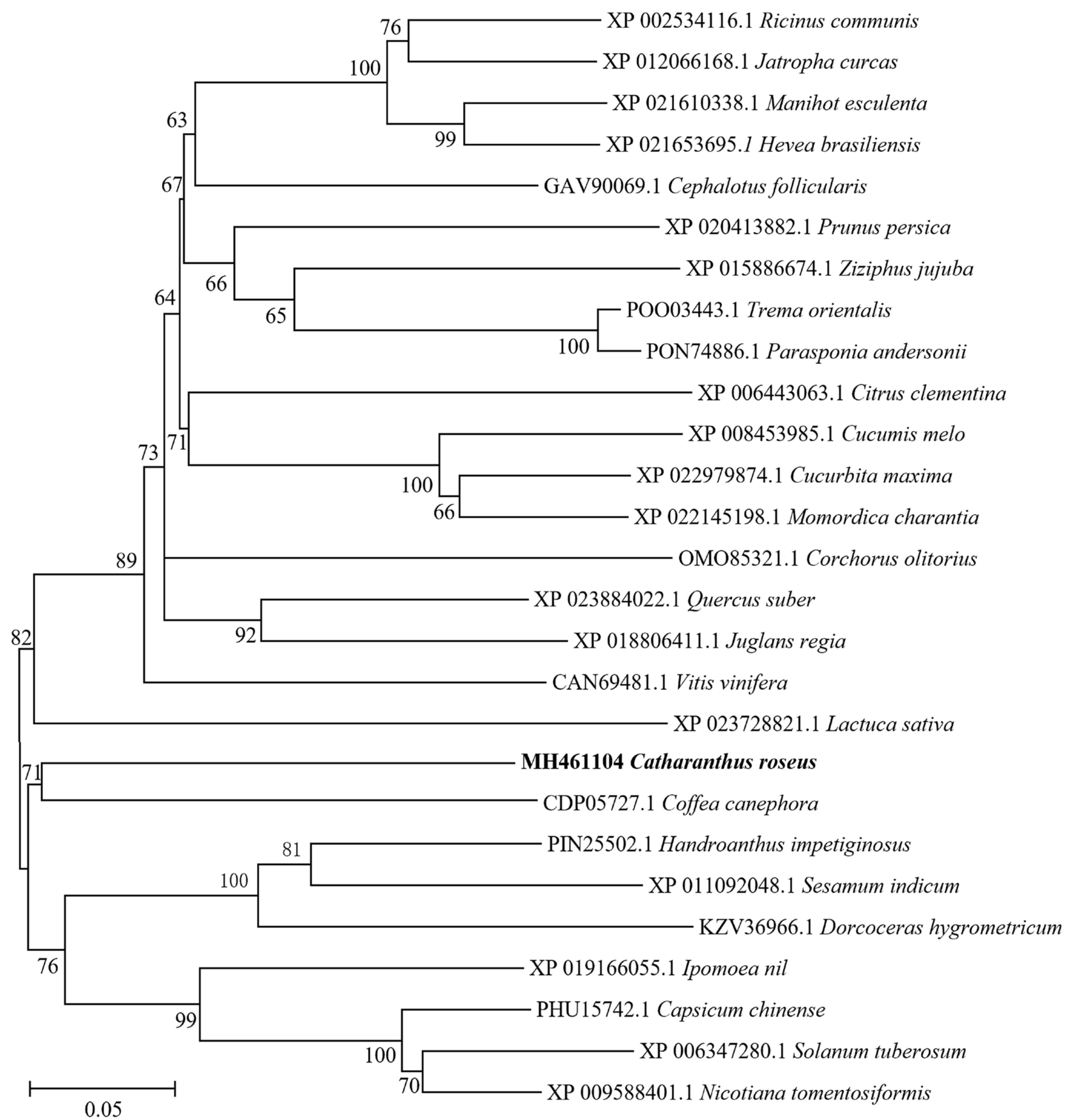

Figure 2. A neighbour-joining phylogenetic tree showing the evolutionary relationships between CrFTA and the sequences of the other predicted CrFTA proteins from 26 angiosperm species

The numbers shown on the branch nodes represent the bootstrap values; CrFTA from *C. roseus* is shown in bold

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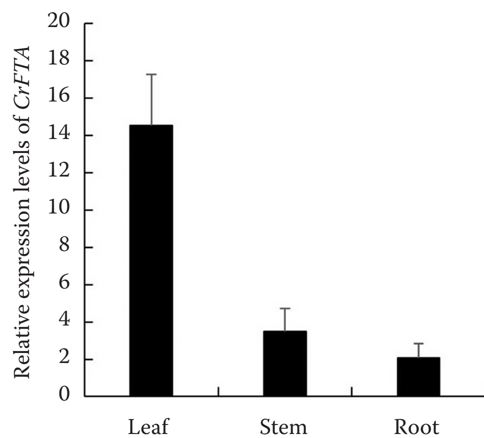

Figure 3. Relative gene expression of *CrFTA* in the leaves, stems, and roots of the healthy *Catharanthus roseus* plants; the expression of *CrFTA* was normalised to the expression of *18S rRNA*

Expression analysis of *CrFTA* in different tissues. The qRT-PCR was performed to analyse the expression levels of *CrFTA* in three different tissues of the healthy *C. roseus* plants (Figure 3). The results showed that the *CrFTA* mRNA is present in the leaves, stems, and roots. The relative expression level of *CrFTA* in the leaves is ~4-fold and ~7-fold higher, respectively, when compared to that in the stems and roots.

Expression pattern of *CrFTA* in *C. roseus* plants infected with *Ca.* L. asiaticus. The expression pattern of *CrFTA* in the leaves of the *Ca.* L. asiaticus-infected plants from 0 to 32 DAT is shown in Figure 4. The *CrFTA* expression levels were found to be significantly upregulated, starting at 4 DAT, and the effect was especially pronounced in groups I and II. In the *Ca.* L. asiaticus-positive plants after top-grafting, the *CrFTA* expression levels increased rapidly, peaking at 22 DAT in groups I and II an at 24 DAT in groups III and IV. The relative expression of *CrFTA* then decreased gradually, but was still higher than the expression levels in the CK group until the end of the experiment at 32 DAT. The highest expression levels in groups I–IV were ~18-fold, 17-fold, 9-fold, and 8-fold higher than the CK group, respectively. Furthermore, the *CrFTA* mRNA levels in groups I and II were significantly higher than in groups III and IV from 10 to 28 DAT. There were no statistically significant differences ($P > 0.05$) in the *CrFTA* transcript levels between group I (ethanol only, no ABA) vs. group II (ABA) or between group III (ManuA, no ABA) vs. group IV (ManuA+ABA). There was, however, a significant difference in the *CrFTA* transcript levels between groups III or IV (ManuA) vs. groups I or II (no ManuA) (Figure 4).

Detection of *Ca.* L. asiaticus in *C. roseus* leaves by PCR. PCR was used to detect *Ca.* L. asiaticus in the leaves of all the plants in each group every two days from 10 to 32 DAT, and the results are shown in Figure 5. After top-grafting with the *Ca.* L. asiaticus-infected shoots, the *C. roseus* plants became gradually infected by *Ca.* L. asiaticus, and the pathogen was detected from 12 DAT in groups III and IV and

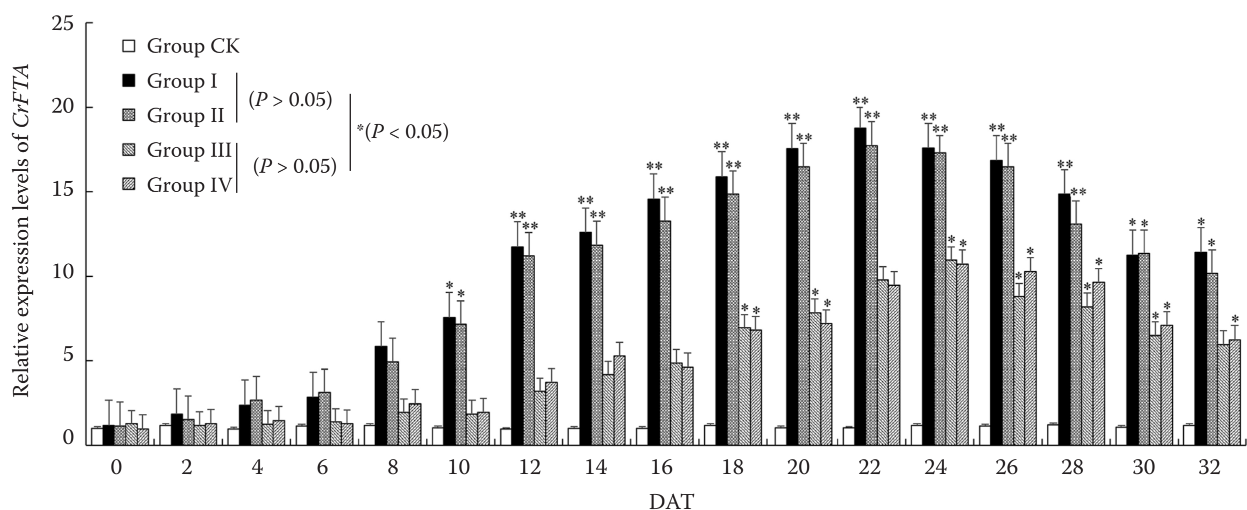

Figure 4. Temporal expression of *CrFTA* in the *Catharanthus roseus* leaves during the *Candidatus* Liberibacter asiaticus (*Ca.* L. asiaticus) infection

The data are expressed as the mean fold-change relative to the ratio of *18S rRNA* expression; the significant differences are indicated by asterisks, *$P < 0.05$, **$P < 0.01$

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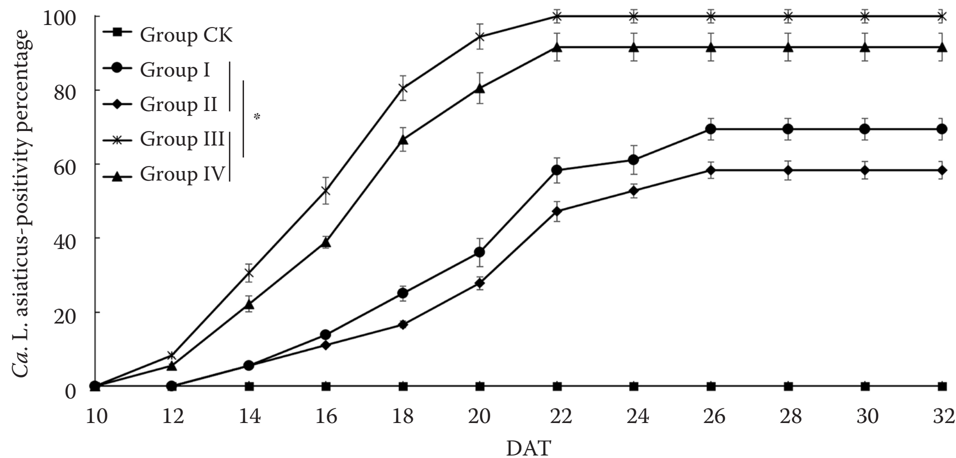

Figure 5. The *Candidatus* Liberibacter asiaticus (*Ca*. L. asiaticus) positivity percentage in the *Catharanthus roseus* plants after top-grafting with the *Ca*. L. asiaticus-infected shoots

The *Ca*. L. asiaticus-positive percentage = the number of *Ca*. L. asiaticus-positive plants/the total number of experimental plants × 100%; the significant differences between the groups are indicated by asterisks, *P <0.05

14 DAT in groups I and II. Furthermore, the *Ca*. L. asiaticus positivity percentage increased significantly in all the experimental groups, and the final positivity percentages reached 69.4%, 58.3%, 100%, and 91.7% for groups I, II, III, and IV, respectively. There were no statistically significant differences between group I vs. group II or group III vs. group IV, but there were significant differences between group III or IV vs. group I or II.

DISCUSSION

Some studies have shown that farnesyltransferase plays an important role in ABA signalling in plants (Nambara & McCourt 1999). In the present study, the gene for the farnesyltransferase α-subunit (FTA) homologue CrFTA was identified in *Catharanthus roseus*, the Madagascar periwinkle. CrFTA contains a predicted PPTA motif, and could, therefore, recognise proteins containing the CaaX consensus sequence (Izard 2002). Proteins that contain the CaaX consensus sequence can be modified and change the solubility, compartmentalisation, and interaction with other proteins through the covalent attachment of farnesyl moieties by farnesyltransferase (Goritschnig et al. 2008). The predicted CrFTA protein clusters in a clade with the FTA protein sequences from diverse species in the subclass Asteridae, and showed the closest evolutionary relationship with the FTA from *Coffea canephora* in our phylogenetic analysis. The qRT-PCR results suggest that the expression of *CrFTA* is constitutive and ubiquitous because *CrFTA* was detected in all of the tested tissues. This broad expression pattern of *FTA* has also been detected in other plant species, such as oilseed rape (Wang et al. 2009). The highest relative expression levels of *CrFTA* were detected in the leaves of the *C. roseus* plants, but the highest expression was found in the roots of the oilseed rape (Wang et al. 2009). These results suggest that the *FTA* gene expression patterns may differ in different plant species.

A plant's defence against pathogens requires a complicated cellular signalling network. The localisation of defence signalling components and their interaction with other proteins are prerequisites for plant defence responses, and these usually depend on post-translational modifications through the PPTA motif of some proteins combining with the CAAX consensus sequence in the defence signalling proteins (Charng et al. 2014). In this study, the conserved PPTA motif was detected in the predicted CrFTA protein. This suggests that some CAAX proteins modified through prenylation by CrFTA may facilitate localisation in cellular membranes and recruit some defence signalling molecules. The *CrFTA* gene expression levels were significantly increased during the *Ca*. L. asiaticus infection, suggesting that CrFTA can be activated to participate in the defence responses against pathogens.

Previous studies have demonstrated that farnesyltransferase plays an important role in tolerance to unfavourable environmental conditions through the ABA signalling pathway (Goritschnig et al. 2008). When treated with the specific farnesyltransferase inhibitor ManuA, the *CrFTA* expression levels in the *C. roseus* leaves were significantly suppressed

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and the *Ca*. L. asiaticus positivity percentage in the plants was significantly increased after top-grafting. This result suggests that CrFTA may play an important role in the pathogenesis and defence responses to *Ca*. L. asiaticus in *C. roseus*. Furthermore, we found that ABA had no significant effect on changing the *CrFTA* expression levels and the numbers of the *Ca*. L. asiaticus-positive *C. roseus* plants after top-grafting. The results of our study suggest that CrFTA plays an important role in mediating resistance to a Ca. L. asiaticus infection in *C. roseus*. The results will contribute to the further understanding of the function of FTA in a plant's immunity and the development of effective strategies for controlling *Ca*. L. asiaticus in citrus.

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