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Full Length Research Paper

Cloning and expression of cell wall acid invertase gene fragment from poinsettia (*Euphorbia pulcherrima* wild.)

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A fragment of invertase gene containing catalytic sites of cysteine was cloned from poinsettia (*Euphorbia pulcherrima* wild.) by using the polymerase chain reaction (PCR) method. The length of the fragment was 521 bp, encoding 173 amino acids and containing a part of open reading frames, but no intron. It had a high homology to previously cloned cell wall acid invertase genes in other plants by sequence comparison, so it could be speculated that the fragment belongs to putative cell-wall acid invertase gene sequence of poinsettia (termed as *EpCWINV*, GenBank no. EU274662). Gene expression analyses in different organs including roots, stems, leaves, flowers among others and during different growth periods of leaves by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method and real-time quantitative PCR approach revealed the highest level of expression in roots, the lower in stems and intermediate in leaves and flowers, as well as a continuous rising level in leaves and a continuous declining level in petioles during the developmental stages of the leaves.

Key words: Poinsettia, invertase gene, cloning, gene expression, RT-PCR.

INTRODUCTION

Sugar metabolism, translocation and sensing (notably sucrose) play a critical role in plant growth and development (Lalonde et al., 2004; Rolland et al., 2006). Its control is influenced by a variety of regulatory mechanisms, among which invertases show a crucial contribution. Invertases, which are responsible for catalyzing the hydrolysis of sucrose into glucose and fructose, are usually divided into acid, neutral or alkaline groups according to the optimum pH requirements and subcellular localization. Acid invertase exists in the apoplast (insoluble) and the vacuole (soluble), whereas neutral or alkaline invertase appears to be located in the cytoplasm (Roitsch and Gonzalez, 2004). It is generally accepted that invertases are involved in modulating partitioning of photosynthetic products, plant development, hormone responses and stress responses and therefore shape all aspects of plant life cycle, including fruit quality, yield and fertility. (Roitsch and Weber, 2000; Roitsch and Gonzalez, 2004). Accordingly, increasing attention was paid to invertases, as yet a great number of plant invertase genes were cloned and analyzed from numerous plants, such as *Arabidopsis thaliana* (Tymowska-Lalanne and Kreis, 1998), barley (Nagaraj et al., 2005), sugar beet (Gonzalez et al., 2005; Gonzalez and Cejudo, 2007) and sorghum (Jain et al., 2008).

Poinsettia (*Euphorbia pulcherrima* wild.) is one of the most popular potted flowers in the world. Its prominent ornamental feature is a bract, a kind of red abnormal leaf, which is born on the top of the plant rather than the flower. However, studies on the physiological mechanism of sugar metabolism and related genes are few in poinsettia. In the present study, the cell wall acid invertase gene fragment from the poinsettia cultivar 'Early Velvet' was cloned and investigation was carried out on its expression profiles in roots, stems, leaves, flowers and bracts during different developmental stages by reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time polymerase chain reaction (PCR) technology, in an attempt to understand what role this gene would play in the sucrose metabolism during the growth and development of poinsettia.

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MATERIALS AND METHODS

Materials

This experiment was carried out from August to November 2006, in a greenhouse in the Horticultural Institute of Jiangsu Province. Poinsettia cultivar, 'Early Velvet', was grown in culture media (sphagnum: perlite = 8:2). Their trunks were topped 6 - 7 leaves and 4 - 5 collaterals were kept. On 25th September, a number of plants with uniform growing status and 4 - 5 start-leaf on the collateral was chosen as the experimental materials. On the cultural condition, temperature was 21 - 24℃ during the light period and 16 - 18℃ during the dark period. The functional leaves near the bracts at October 31st (early growth stage of bracts) and November 15th (intermediate growth stage of bracts) were taken as materials, respectively. Roots, stems, leaves, bracts and flowers at November 30th (mature stage of bracts) were taken for analysis, The sampling time was around 9:00 am. The samples were flash frozen under liquid nitrogen when harvested and stored at - 80℃ for further use.

Cloning and sequence analysis

Genomic DNA extraction was performed following the methods described by Chen et al. (1997). The DNA quality and concentration were analyzed by electrophoresis and spectrophotometry, respectively, diluted to 25 ng μl^{-1} with TE (Tris + EDTA) buffer and stored at -20℃ until use. Primers were designed according to the conservative domain of putative plant cell-wall invertase: UP1 (5'-TACCATCTATTCTACCAGTACAA-3'), DP2 (5'-AAAAAATCAGG ACACTCCCACAT-3'). The reaction mixtures were as follows: 50 ng genomic DNA, 0.2 µM UP1 and DP2 respectively, 1×PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs and 2 U Taq DNA polymerase (Takara, Japan) in a final volume of 50 µl. The programs for PCR were as follows: Preamplification denaturation at 95℃ for 5 min, 30 cycles of (denaturation at 95℃ for 40 s, annealing at 50℃ for 40 s, extension at 72℃ for 1 min) and a final extension of 72℃ for 10 min, and then stored at 4℃. The PCR products were electrophoresised in 1% agarose gel. The PCR products were purified and recovered for ligation reaction using a purification reagent kit (Sangon). Then the purified DNA fragment was ligated into the pMD 18-T cloning vector (TAKARA) and transformed into the competent cells of *Escherichia coli* DH-5α strain. The recombinants were screened from non-recombinants using ampicillin and identified through restriction enzyme digestion of *Sal* I and *Xba* I. The clones were sequenced in both directions with an ABI DNA analyzer. The obtained sequence was analysed by DNAMAN and Clustalx software.

RNA isolation semiquantitative RT-PCR analysis

Total RNA of poinsettia was isolated according to the method described by Xu et al. (2004). The first strand of cDNA was synthesized by using SYBR ExScript™ RT-PCR Kit reverse transcription reagent box (TaKaRa company). According to the cloned fragments sequence (EpCWINV), the gene-specific primers were designed. The upstream primer was 5'-ACGCTCTTTACCCATCAG-3' and the downstream primer was 5'-TCAGGACACTCCCACATC-3' while the predicted amplified fragment was 412 bp in length. The gene *OsActin* was used as a control. The amplifed product of OsActin gene contains an intron. Its upstream primer was 5'-CTGGGTT CGCCGGAGATGAT-3' and downstream primer was 5'-TGAGAT CACGCCCAGCAAGG-3' while its amplified fragment was 501 bp in length. The composition of the reaction mixture was as follows: 2 µl of cDNA, 0.2 µM primer mixture (OsActin as the internal control), 1 × PCR buffer (including $MgCl_2$), 200 µ mol/L dNTPs and 2 U of Taq DNA polymerase in a final volume of 25 µl. The programs for PCR were the same as described above. The amplified products were electrophoresised in 1% argarose gel and photographed under UV light.

Real-time quantitative PCR analyses

Real-Time PCR analysis was carried out by SYBR Green I chimeric fluorescence method (ABI PRISM 7500 real-Time PCR System). The data were collected using 7500 system sequence detection system software version 3.0. The experiment was operated in accordance with the instrument manual of Applied Biosystems Company. The preparation of PCR reactive solution was operated in accordance with the manual of SYBR ExScript™ RT-PCR Kit PCR reagent kit (TaKaRa company) and contained 2× SYBR Premix Ex Taq 10.01 µl, 50 × ROX Rference Dye II 0.4 µl, 1 µl cDNA solution as a template, 0.4 µl mix solution of target gene primer (with OsActin as internal control) in a final volume of 20 ml, each having four replicates. The specific amplification procedure was as follows: 50℃ for 2 min; 95℃ for 10 min; 40 cycles of 95℃ for 15 s, 60℃ for 1 min. A melting curve analysis was performed after amplification. The quantitative results were automatically calculated by 7500 system sequence detection software (version 3.0). In order to determine the quantitative results, the relative expression level in the tissue (the trunk between red leaf to semi-red leaf in colouring stage) possessing the lowest mRNA expression level of cell wall acid invertase mRNA was set as 1.

RESULTS

Cloning and sequencing

Using poinsettia genomic DNA as a template, a specific band (approx. 500 bp) was obtained through PCR amplification with the specific primers designed (Figure 1) and the recombinant plasmid sequence that possess such fragment characterized by double-digestion with SalI and XbaI (Figure 1) was sequenced and subsequently analyzed by DNAMAN software. The results showed that the cloned fragment was 521 bp in length, encoding 173 amino acids and belonging to a part of open reading frame, but no intron (Figure 2). Additionally, there is conserved cysteine catalytic motif WECPD (underlined in Figure 2) that presents in all plant cell-wall invertases (CWIs) identified so far (Roitsch and Gonzalez, 2004). Systematic BLAST searches among the GenBank databases revealed that the obtained fragment has relatively high similarities with sequences encoding plant CWIs. The sequence has 62, 61 and 60% amino acids identity with the invertase of chicory (Y11124), Pea (AF063246) and papaya (AF420223), respectively. Therefore, it could be speculated that such sequence is a part of putative poinsettia cell wall acid invertase gene, which is designated as *EpCWINV*.

Expression of cell wall acid invertase gene in different organs of poinsettia

Figure 3 shows that the cell wall invertase gene was expressed in all test organs at the matured stage of poinsettia bracts, but at different expression levels. The

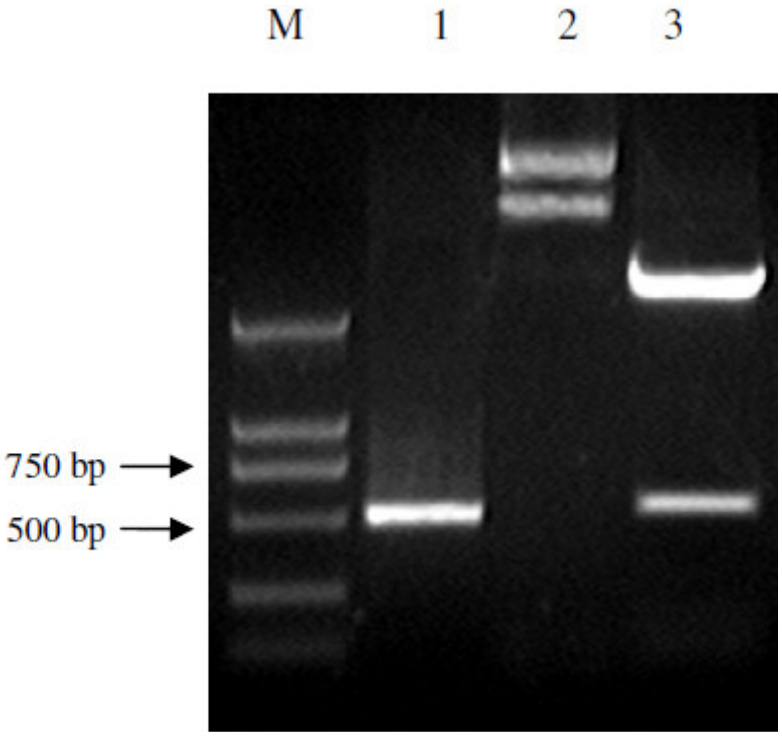

Figure 1. Cloning of a positive fragment (*EpCWINV*) and its characterization by restriction digestion. M, 1, 2, 3 are DL2000 marker, PCR product, pMD-18T-EpCWINV, pMD-18T-EpCWINV disgested with SalI and XbaI, respectively.

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1    taccatctattctaccagtacaatccaaagggtgctgtatttggtgatcaaatggtatgg
1     Y  H  L  F  Y  Q  Y  N  P  K  G  A  V  F  G  D  Q  M  V  W
61   gctcattctgtatcatatgatctcatcaattgggttcgtctcaaacatgctctttatcca
21    A  H  S  V  S  Y  D  L  I  N  W  V  R  L  K  H  A  L  Y  P
121  accgaaccattcgacatcaacagttgctggtctggttctgttacaatacttcccggtaat
41    T  E  P  F  D  I  N  S  C  W  S  G  S  V  T  I  L  P  G  N
181  aaacctgccattttatacaccggaatagatgctaaccatacacaggttcagaatttagct
61    K  P  A  I  L  Y  T  G  I  D  A  N  H  T  Q  V  Q  N  L  A
241  gtccctaagaatctctctgacccttactattagaatgggtcaagctttcgggaaatccc
81    V  P  K  N  L  S  D  P  L  L  L  E  W  V  K  L  S  G  N  P
301  gttatggtccgacccagtggagtcaaccgcgatgactttcgagaccccacaaccgcttgg
101   V  M  V  R  P  S  G  V  N  R  D  D  F  R  D  P  T  T  A  W
361  ctcggtcctgatggcaaatggaatgtgattgttggtggaaaattgaataatcgaggaatc
121   L  G  P  D  G  K  W  N  V  I  V  G  G  K  L  N  N  R  G  I
421  gctttttgtatcaaagtgttgactttgttaactggactaagcacgaaaatcctctttat
141   A  F  L  Y  Q  S  V  D  F  V  N  W  T  K  H  E  N  P  L  Y
481  tcggtggaacaaactgggatgtgggagtgtcctgatttttt
161   S  V  E  Q  T  G  M  W  E  C  P  D  F
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Figure 2. Sequence analysis of *Euphorbia pulcherrima* invertase gene fragment. The nucleotide sequence and derived amino acid sequence of *EpCWINV* are shown. The cysteine catalytic motif is underlined.

highest expression level was found in roots, lower in stems and bracts. At the same time, only a single fragment of 500 bp involved in actin gene was amplified, which revealed that the removal of genome DNA from RNA was thorough and the available cDNA meets the requirement of real-time quantitative PCR analysis. In

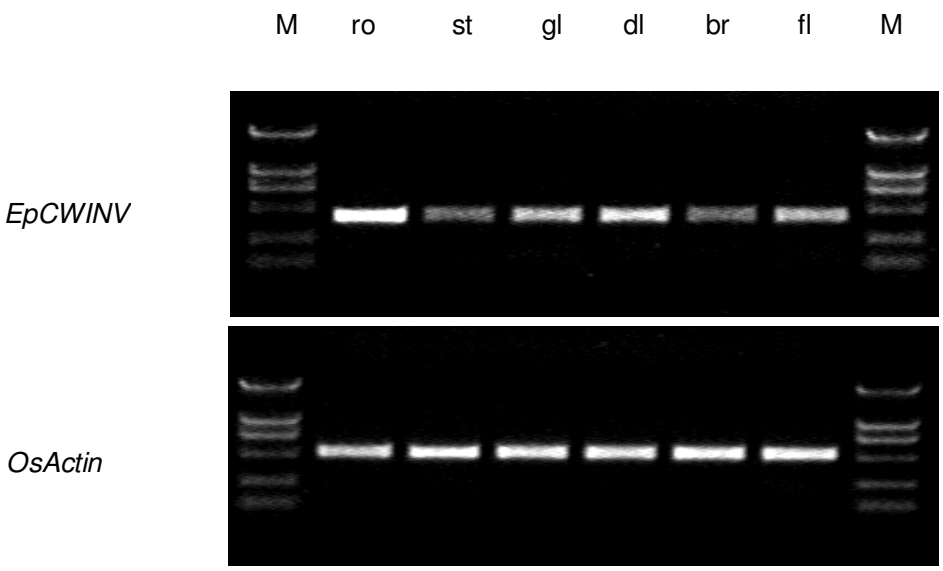

Figure 3. Expression pattern of *EpCWINV* and *OsActin* in different organs at the mature stage of poinsettia bracts by RT-PCR analysis. M, ro, st, gl, dl, br, fl are DL2000 marker, roots, stems, green leaves, discoloured leaves, bracts, flowers, respectively.

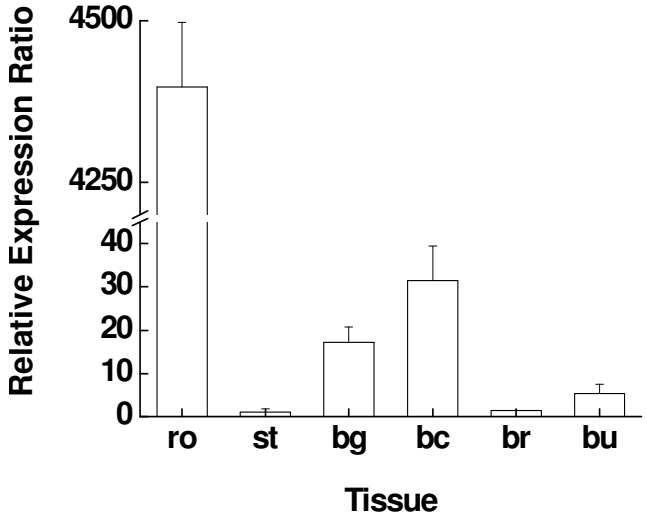

Figure 4. Relative expression ratio of *EpCWINV* in different organs at the mature stage of poinsettia bracts by real-time quantitative PCR analysis. Ro, st, bg, bc, br, bu are roots, stems, green leaves, discoloured leaves, bracts, flowers, respectively.

order to determine the exact expression quantity of cell-wall invertase, a real-time quantitative PCR analysis was performed. As showed by Figure 4, the relative expression ratio of *EpCWINV* in roots were the highest at 4397.18, which is far more than in other organs; the relative expression ratio of *EpCWINV* in green leaves and discoloured leaves were 17.19 and 31.44, respectively, whereas only 1, 1.44 and 5.33 in stems, bracts and flowers, respectively. These data were in agreement with RT-PCR analysis.

Expression of cell wall acidic invertase gene in different developmental stages of poinsettia bracts

It could be seen that the expression levels of *EpCWINV* in leaves and petioles were close on October 31st. At that moment, bracts did not develop. The expression levels in leaves were up regulated while the expression levels in petioles were down regulated on November 15th and November 30th (Figure 5). The result of Figure 6 reconfirmed that the transcript levels of *EpCWINV* in leaves were gradually increased, while a decreasing trend was found in petioles during the developmental process of the bracts.

DISCUSSION

Invertases contribute to all aspects of plant growth and development, especially the regulation of source/sink metabolism (Weber et al., 1996; Sherson et al., 2003; Ishimaru et al., 2005; Jain et al., 2008). There have been many reports focused on invertase molecular biology, ranging from its gene cloning and expression to its transgenic studies. Recent studies elucidated that the invertase genes were composed of a multi-gene family in any given plant for example, vacuolar and cell wall-bound isoforms were represented by more than one gene (Coupe et al., 2003). In this study, only one cell-wall acid invertase gene fragments from poinsettia was isolated in that no other invertase genes not found may be related to simple PCR amplification depended on specific primer design.

Cell-wall invertase genes are expressed in a development and organ-specific manner in various plants. Lorenz

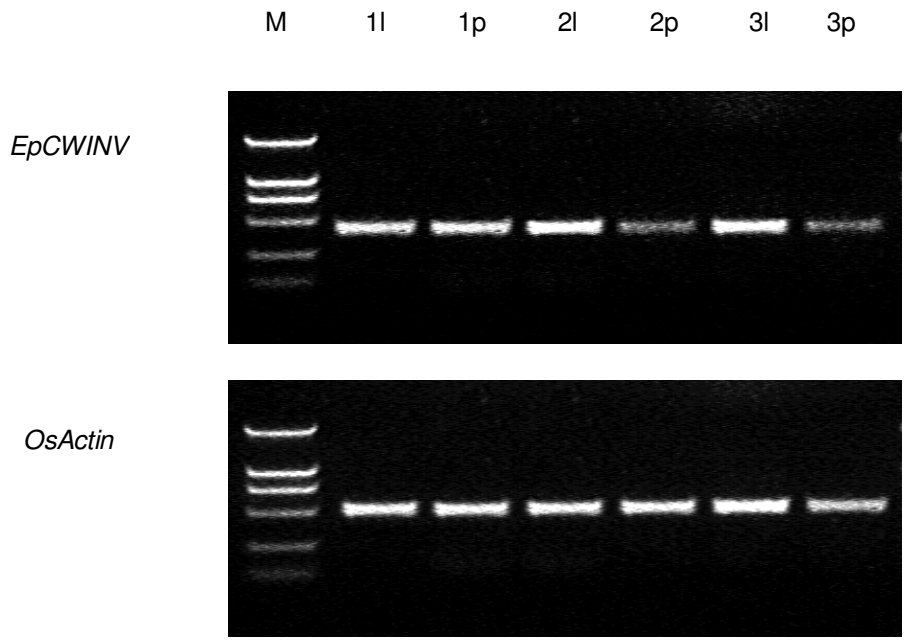

Figure 5. Expression pattern of *EpCWINV* and *OsActin* in different developmental stages of poinsettia leaves by RT-PCR analysis. M, 1l, 1p, 2 l, 2 p, 3 l, 3 p are DL2000 marker, leaves of October 31th, petioles of October 31st, leaves of November 15th, petioles of November 15th, leaves of November 30th, petioles of November 30th respectively.

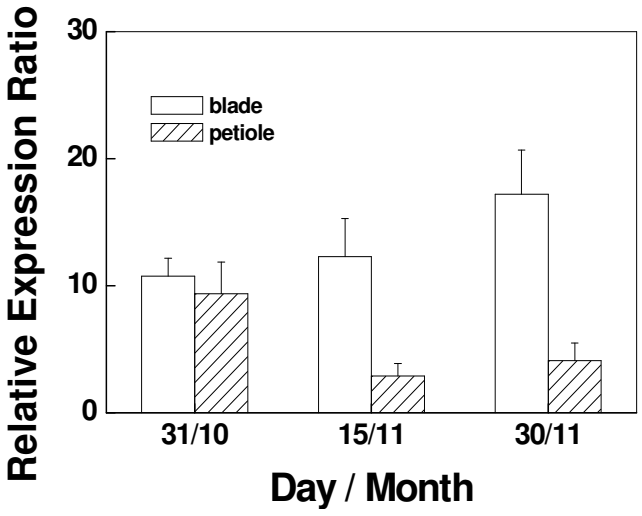

Figure 6. Relative expression ratio of *EpCWINV* in the leaves (represented by blade in figure) and petioles at different developmental stage of poinsettia bracts by real-time quantitative PCR analysis.

et al. (1995) found that the expression patterns of three cell wall invertase gene Inv*Dc1, Inv*Dc2, Inv*Dc3 were significantly different in carrot. The transcripts in the young leaves and roots were of a high level, while transcripts were not detected in plants with developing tap roots. Inv*Dc2 and Inv*Dc3 transcripts were not present in vegetative plant organs. Corn also has four cell wall invertase genes. Incwl, Incw2, Incw3 and Incw4 and the expression of Incwl, Incw2 showed organ-specific manner (Taliercio et al., 1999). In the present study, EpC WINV of poinsettia was constitutively expressed, but the transcript level differed markedly in different organs. RT-PCR and real-time quantitative PCR analysis showed that EpCWINV had a well high expression level in roots, the normal level in green leaves and discoloured leaves and a low level in the red bracts, stems and flowers. In view of this, it could be concluded that such gene has great effect on the assimilation of roots at the mature stage of poinsettia bracts.

Likewise, EpCWINV also plays an important role in green leaves, the expression level of which was enhanced during the colouring of bracts, which sharply decreased when all the leaves turned into red. The red bracts had a relatively low expression level in the middle stage of bracts formation, the relative expression ratio of which is only 2.25. However, the expression of this gene showed a continuous rising level in green leaves and transitional leaves and a declining level in petioles during the development of bracts, indicating that EpCWINV may play a role in the enhancement of sink strength for leaves. Furthermore, studies on cell-wall invertase activity of poinsettia showed that high CWI activity was found in developing bracts (unpublished data), which reaffirms the important role of the gene in the early establishment of sink strength.

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