Survey on Gene Expression of Phytocystatin from Local Taro (*Colocasia esculenta)* Plants

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ABSTRACT

An investigation on the cystein protease inhibitor (cystatin) activity of the local taro (*Colocasia esculenta* (L.) Schott.) cultivars was carried out. Interestingly, the cultivar Kaohsiung No. 1 showed great positive and Ch'ih-Ya-Yu showed little activity. The other two cultivars, i.e., Pin-Lang-Hsin and Kou-Ti-Yu, never showed the inhibitory activities. Southern gel blot revealed that more than one copy of cystatin gene was present in the haploid genome of Kaohsiung No 1 cultivar. This result indicated that cystatin gene can be an useful marker to differentiate Kaohsiung No 1 cultivar from others. Further studies demonstrated that cystatin genes are differentially expressed in corm organ, and more abundant in the periderm tissue of corm. They are able to retain active expression for four weeks long during storage condition. Therefore, the significance of cystatin gene expression is considered to be closely related with the resistance of pest invasion.

Key words: Cysteine proteinase inhibitor, Taro, Corm, Periderm.

芋頭中植物性半胱氨酸蛋白水解酶抑制劑之基因表現

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摘要

針對臺灣地區四種芋頭 (*Colocasia esculenta* (L.) Schott.)栽培品系進行半胱氨酸蛋白酶抑制劑 (cystatin; cysteine proteinase inhibitor)的測試調查，首先利用明膠凝膠活性電泳測試法 (gelatin/SDS-PAGE)分析四種芋頭球莖中的蛋白質粗抽取液，其中高雄一號(Kaosiung No.1)具有強烈的半胱氨酸蛋白水解酶抑制劑的活性，及赤芽芋(Ch'ih-Ya-Yu)有一點活性之外，其他二種品系狗蹄芋(Kou-Ti-Yu)及檳榔心(Pin-Lang-Hsin)則不具此活性。以南方轉印法分析此基因拷貝數，發現高雄一號基因組(genome)中含有一個以上的基因拷貝數(gene copy)；北方轉印的分析顯示，此基因只特定表現於芋頭球莖中，尤其在球莖的周皮組織(periderm)特別豐富，其他組織如葉部及葉柄皆不表現；即使採收後的球莖儲存至第四週此基因仍有極強表現量。從這些調查結果顯示，雖然芋頭的 cystatin 基因其演化來源未知，但卻可作為分辨高雄一號品系的 DNA marker，其在高雄一號中獨特存在及豐富的表現量，可能是此品系較其餘三種品系具有抗病蟲力的主要原因。

關鍵詞：半胱氨酸蛋白酶抑制劑、芋頭、球莖、周皮層。

INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott), a member of the family *Araceae*, is one of the major starchy root

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投稿日期：2004 年 12 月 27 日
接受日期：2005 年 1 月 18 日
作物、環境與生物資訊 2:123-130 (2005)
Crop, Environment & Bioinformatics 2: 123-130 (2005)
189 Chung-Cheng Rd., Wufeng, Taichung Hsien 41301, Taiwan (ROC)

crops in Asia and Oceania. The mature corm of taro contains about 1.4-3.0 % protein on a fresh weight basis (Purseglove 1972). Storage protein offered an interesting model system to study the regulation of gene expression during development and with respect to organ specificity. They are present in storage organs and sometimes can be detected in small amounts in other plant organs and tissues. These proteins may function as proteinase inhibitors, and protect plants from damage by insects and pathogen.

Protease inhibitors are grouped into families according to their specificity: serine-protease, cysteine-protease, aspartate-protease, and metallo-protease inhibitors (Terra and Ferreira 1994). Most of the earlier research focused on Lepidopteral and Dipteral orders that generally use serine proteases to digest proteins and their mechanism of trypsin inhibition is based on competition through the blocking of proteolytic activity. More recent studies have shown that ranges of other classes of proteases are also found in insect guts (Wolfson and Murdock 1990). Proteins capable of selective and specific inhibition of cysteine proteinase have been identified as cystatins and are isolated from a variety of microbes and tissues of animals and plants.

Cystatins inhibit sulfhydryl proteinase activities and have mainly been studied in animal cells. Animal cystatins have been analyzed and classified into 3 families based on their molecular structures. Family-1 cystatins (stefins) are about 100 aa long with no disulfide bonds, family-II (cystatin II) are about 150 aa long with two disulfide bonds, and family-III cystatins (the kiminogens) contain three segments homologous to the family-II cystatins (Barrett 1987). The cysteine proteinase inhibitors from plants that show amino acid sequence similarity to the animal cystatins are referred to as the phytocystatins. Oryzacystatin-I (OC-I) (Abe *et al.* 1987a) and oryzacystatin-II (OC-II) (Komdo *et al*. 1990), which were discovered from rice, are the first two well-defined cystatins of plant origin. Phytocystatins resemble the stefins in lacking disulfide bonds and carbohydrates and having a molecular mass of ~11 kDa and share three conserved sequence motifs (Abe *et al*. 1987, Kondo *et al*. 1990, Femandes *et al*. 1993). They are abundant in plants and have been identified in seeds (Abe *et al*. 1987, Hirashiki *et al*. 1990, Abe and Arai 1991; Kouzuma *et al*. 1996, Li *et al*. 1996, Ojima *et al*. 1997, Brzin *et al*. 1998, Pernas *et al*. 1998, Rassam and Laing 2004), leaves (Zhao *et al*. 1996, Gaddour *et al*. 2001), tubes (Waldron *et al*. 1993), roots (Gaddour *et al*. 2001), fruit (Kimura *et al*. 1995; Ryan *et al*. 1998, Rassam and Laing 2004) and floral buds (Lim *et al*. 1996). While most commonly they are small proteins ranging from 11 to 16 kDa (Laing and McManus 2002), a few cystatins are significantly larger (Joshi *et al*. 1998, Misaka *et al*. 1996, Siqueira-Junior *et al*. 2002, Waldron *et al*. 1993). Phytocystatins show variable expression patterns during plant development and defense responses to biotic and abiotic stresses (Felton and Korth 2000). The physiological function of these proteins is not well understood. However, at least two functions have been proposed: regulation of protein turnover and protecting plants against insects and pathogens (Turk and Bode 1991).

Previously, we isolated a cDNA gene from mature taro corm. It was characterized and its protein was identified with strong cysteine protease inhibitory activity. Here we report the investigation on all local taro cultivars and the expression pattern of the cystatin gene in corm of Kaohsiung No. 1 cultivar. The quantitative presence of cystatin gene in Kaohsiung No.1 and Ch'ih-Ya-Yu are interesting and considered to be related with its high resistance to pest infection.

MATERIALS AND METHODS

PLANT MATERIALS

The taro plants were maintained at the experiments farm of Kaohsiung District Agricultural Improvement Station, Pingtung, Taiwan. The tissues were harvested and freshly sliced, frozen in liquid nitrogen, and stored in –70℃ until the use of protein extraction.

PROTEIN EXTRACTION AND PURIFICATION

Taro plant tissues were ground in liquid nitrogen and homogenized in 100 mM sodium phosphate buffer, pH 6.0, containing 1 mM DTT and 1 mM EDTA. The homogenate was heated at 80℃ for 10 min to inactive endogenous proteinases. The crude extract was centrifuged at 10,000 xg for 15 min and the supernatant was subjected to 80% ammonium sulfate fraction. The fraction was against 50 mM sodium acetate buffer (pH 5.2), and then stored at –20℃. Protein concentrations were determined according to Bradford (Bradford 1987) with bovine serum albumin as a protein standard.

ELECTROPHORESIS ANALYSIS

For inhibitory activity assay, 100 μg of partial puried protein sample (cystatin) was incubated with 2 μl of 0.25 mM papain for 15 min at 37℃. After incubation, 6 μl of mildly-denaturing gelatin-page sample buffer (62.5 mM Tris-HCl, pH 6.8, 2 % w/v SDS, 2 % w/v sucrose, 0.001 % w/v bromophenol blue) was added to mixture, and were resolved on 10% w/v SDS-polyacrylamide slab gel containing 0.1% w/v gelatin (Michaud *et al*. 1996). To avoid migration of the gelatin out of the resolving gel, the ratio of acrylamide to N,N'-methylenebisacrylamide (bis) was adjusted to 29.0 : 1.1, and the migration was performed at 4℃. Protein samples were first subjected to electrophoresis using a Hoefer SE 250 system. After migration, the gels were transferred to a 2.5% v/v aqueous solution of triton X-100 for 30 min at room temperature to allow renaturation, then incubated at active buffer (100 mM sodium phosphate pH 6.8; 8 mM EDTA; 10 mM L-cysteine and 0.2% triton X-100) for 75 min at 37℃. Subsequently, they were rinsed with water and stained with Coomassie brilliant blue (0.1% w/v Coomassie brilliant blur in 25% v/v isopropanol / 10% v/v acetic acid). Proteinase inhibitory activity was visualized as clear zones in a blue background.

QUANTITATIVE ANALYSIS OF THE CYSTEINE PROTEINASE INHIBITORY ACTIVITY OF TARO

As for the quantitative activity assay, the method described by Abe *et al*. (1992) was followed. The 400 μg concentration of protein samples in 0.2 ml were mixed together with 0.1 ml of sodium phosphate buffer (0.5 M sodium phosphate/10 mM EDTA, pH 6.0), 0.1 ml of 2-mercaptoethanol (50 mM), and 0.1 ml of papain solution (25 μg ml^{-1}), and the mixture was incubated at 37℃ for 10 min. After that, 0.2 ml of 1 mM BANA (in DMSO) was added to start the reaction, and the mixture was incubated for another 20 min at 37℃. The reaction was terminated by adding 1 ml of 2% HCl / ethanol and 1 ml of 0.06% p-dimethyl aminocinnamaldehyde / ethanol, and the mixture was allowed to stand at room temperature for 30 min for color development. Finally, OD_{540} nm was measured. The inhibitory activity of cystatin was shown as an inhibition percentage (%) on papain by cystatin. The percent inhibition (I %) of papain by cystatin was calculated using the following equation:

$$I\ \% = [(T-T^*) / T] \times 100\%$$

Where T denotes the OD_{540} in the absence of cystatin and T* is that in the presence of cystatin. One inhibition unit was defined as the amount of inhibitor required to completely inhibit 2.5 μg of papain.

DNA AND RNA PREPARATION AND GEL BLOT ANALYSIS

Total RNA was extracted from mature corms, leaves, roots and petiole following the method described by Yeh *et al*. (1991). For northern blot analysis, samples were resolved on 1.0% agarose gels in the presence of formaldehyde and transferred onto non-charged modified nylon blotting membrane (millipore). Genomic corm DNA for southern blot analysis was following the instruction of the digoxigenin (DIG)-labeled by PCR (Roche). Blots were hybridized with the full-length cDNA of taro cystatin probe (Yang and Yeh, 2005). Hybridization and washing procedures were carried out as per the standard molecular methods (Sambrook and Russell 2001).

RESULTS AND DISCUSSION

Proteinases are involved in a variety of metabolic functions ranging from the control of endogenous metabolism to the degradation of exogenous (foreign) proteins. Most of the endopeptidases that are inhibited by cystatins belong to the papain superfamily: they are cysteine proteinases of *Mr* ~25000 and show sequence homology with papain (Barrett 1987).

In the survey on cysteine protease inhibitory activity of local taro crops, soluble protein extract of 400 μg from corms of four taro cultivars was pre-mixed with 2 μl of papain (0.25 mM), and incubated for 15 min at 37℃, and then resolved on 0.1% gelatin / SDS-PAGE to observe the residual protease activity of papain. Gelatin is easily hydrolyzed by several cysteine proteinases and the hydrolysis of gelatin in SDS/PAGE system is an observation of cysteine proteinase activity (Michaud 1993, 1996). The stability of the gelatin-PAGE system is apparently correlated with the degree of affinity between cysteine proteinase and the inhibitor (Michaud 1996). As shown in Figure 1, the effect of Kaohsiung No.1 almost completely

inhibits papain activity during electrophoresis, and a little activity showed by Ch'ih-Ya-Yu. It indicates that the crude protein extracts of Kaohsiung No.1 and Ch'ih-Ya-Yu contain some kinds of cysteine proteinase inhibitors, therefore, it is able to block papain activity, and no clear zone is observed (Fig. 1, lane 4). On the contrary, the other two cultivars, i.e., Pin-Lang-Hsin and Ko-Ti-Yu, seem to contain no endogenous proteinase inhibitors, therefore, papain activity is so active as to degrade gelatin in gel and form clear zones (Fig. 1, lanes 2, 6 and 8). Furthermore, the inhibitory activity of tarocystatins was quantitatively measured by inhibition percentage (I %) against papain. After reacting each crude protein samples with 0.1ml (2.5 μg) papain. Kaohsiung No.1 had significant inhibitory activity, and Ch'ih-Ya-Yu had a little (Fig. 2). These results showed that Kaohsiung No. 1 of taro could have a cystatin (cysteine proteinase inhibitor) activity in the tissue of corm, while the other two taro cultivars have not. This may be the possible reason that Kaohsiung No.1 has more pest-resistant in the farm and higher productivity than other three cultivars.

To estimate the gene copy number of cystatin gene in Kaohsiung No 1, gel blot analysis of genomic DNA was performed using low stringency hybridization conditions. Taro genomic DNA was digested with *Alu* I, *Bfa* I, *Bag* II, *Eco* RI *and Rsa* I respectively, two bands were clearly hybridized by the Dig-labeled cDNA probe (Fig. 3). These demonstrated that tarocystatin exists more than one copy in the haploid genome of Kaohsiung No 1, while the other three cultivars don't have the homologous gene in the genome (data not shown). Although no signal of Southern blot was found from Ch'ih-Ya-Yu genome, the inhibitory activity was frequently detected from the crude protein samples. It is possibly due to some unknown components that can inhibit papain.

To investigate the expression pattern of tarocystatin mRNA transcripts, the following taro tissues were detected, i.e., small corm cortex, mature corm cortex, periderm, furl leaf, unfurl leaf, and petiole. Northern blot analysis was performed with the same specific probe as in Southern blot analysis. The cystatin mRNA transcripts were specifically detected in periderm and mature corm cortex, but were not detectable from other organs (Fig.4). It demonstrated that cystatin in Kaohsiung No.1 was spatially expressed in corm, especially abundant in peridum tissue (Fig. 4). Likewise, in rice OCII (Kondo *et al*. 1990), maize CCI (Abe *et al*. 1992), wheat WC (Fabienne *et al*. 2002) and chestnut CsC (Pernas *et al*. 1998), the cystatin gene is expressed continuously during grain formation and in mature seeds. It implies that cystatin is usually expressed in sink tissues. On the contrast, the rice OCI mRNA is detected as soon as two weeks after pollination, gradually decreases during seed formation and is not detectable in mature seeds (Abe *et al*. 1987). All of OCI, OCII, WC, and CsC have the ability to inhibit papain in vitro. Results suggest that cystatin could modulate proteinase activity in vivo.

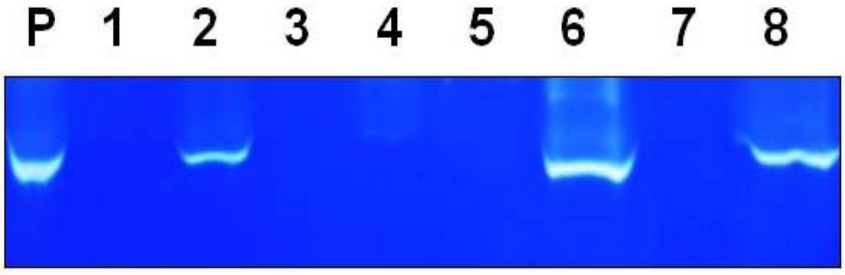

Fig. 1. Gel activity assay of tarocystatin on 0.1 % mildly-denaturing gelatin/ PAGE. Papain was mixed with crude protein extracts of corm from four taro cultivars, and incubated for 15 min at 37 °C, and then submitted to electrophoresis. Each species was loaded with / without 0.25 mM papain 2 μl. P: 0.25 mM papain 2 μl; lane 1 : Chi-Ya-Yu crude extract without papain; lane 2: Chi-Ya-Yu crude extract with papain; lane 3: Kaohsiung No. 1 crude extract without papain; lane 4 : Kaohsiung no. 1 crude extract with papain; lane 5: Pin-Lang-Hsin crude extract without papain; lane 6: Pin-Lang-Hsin crude extract with papain; lane 7: Kou-Ti-Yu crude extract without papain; lane 8: Kou-Ti-Yu crude extract with papain.

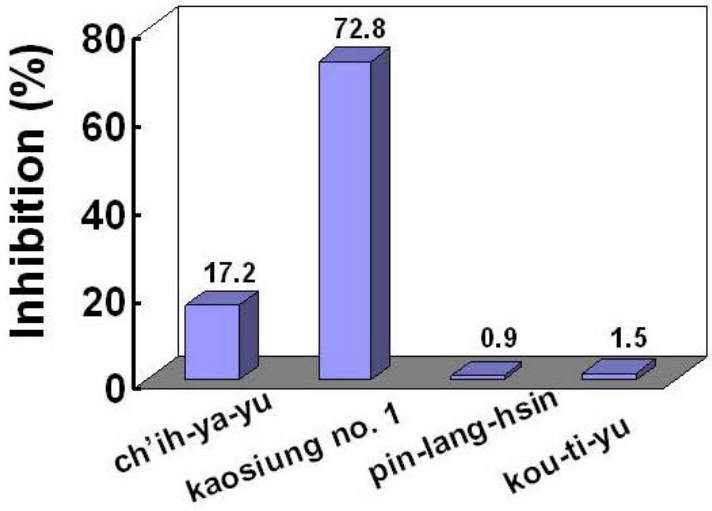

Fig. 2. The endogenous cysteine proteinase inhibitory activities of four taro cultivars against papain. Each crude protein extract of 400 μg was reacted with papain (2.5 μg, Sigma) at 37℃ for 10 min, then measure the residual protease activity of papain.

Fig. 3. Southern gel blot analysis of cystatin gene in cultivar Kaohsiung No 1 genome. Genomic DNA (10 μg) prepared from taro corm was digested with *Alu I, Bfa I, Bag II, Eco RI and Rsa I*, and electrophoresed on a 1% agarose gel. DNA transferred onto a nylon filter and hybridized with Dig-labeled full-length cystatin cDNA insert. The number at the left refers to the positions of DNA molecular length markers in kilobases.

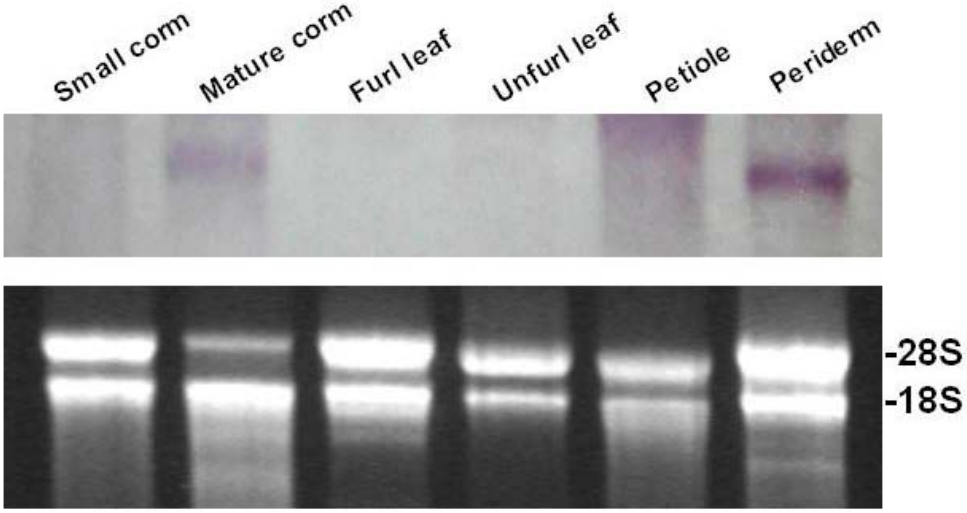

Fig. 4. Northern gel blot analysis of cystatin mRNA transcripts at various tissues. Total RNA of 10 μg isolated from small corm, mature corm, furl leaf, unfurl leaf, petiole and periderm, was resolved on 1% denatured/formaldehyde agarose gel and hybridized with dig-labeled full-length tarocystatin cDNA insert.

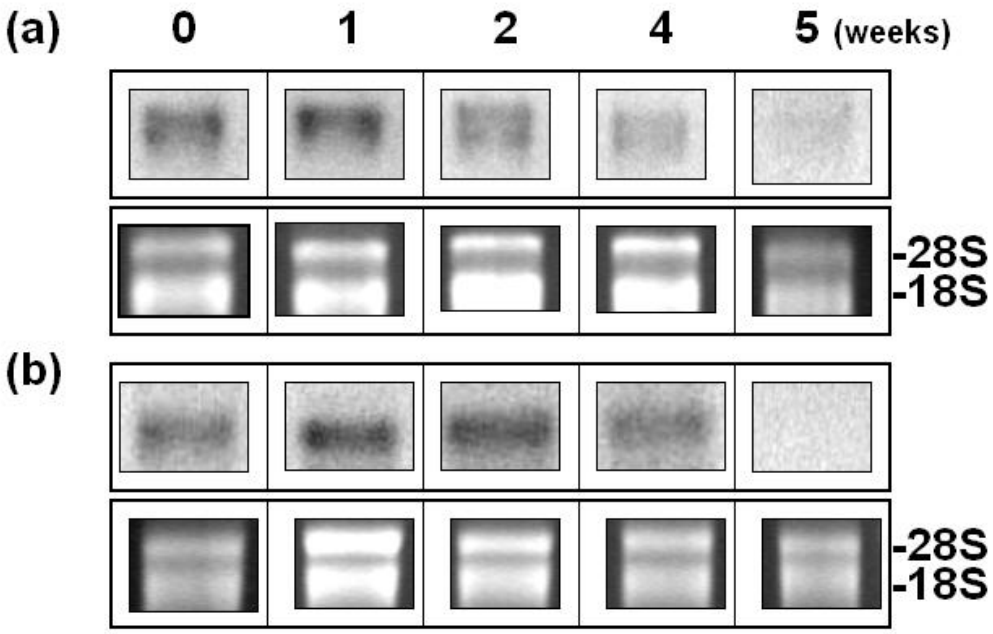

Fig. 5. Northern gel blot analysis of cystatin mRNA transcripts in taro corm during various stored periods (0, 1, 2, 4 and 5 weeks at room temperature). Total RNA of 10 μg isolated from (a) corm cortex without periderm, (b) corm periderm, was probed with the full-length cystatin cDNA insert.

Postharvest stability of tarocystatin mRNA was investigated from corm cortex tissues and corm periderm. Total RNA was prepared from both corm cortex tissues and corm periderm, and were stored from one to five weeks duration in postharvest. As shown in Fig. 5, cystatin mRNA was still actively transcribed during the storage period up to 4 weeks, and the signal intensity declined thereafter. In general, the expression of cystatin in plant seed or organ is considered to be with three different functional roles: (1) regulation of protein turn-over during seed maturation, (2) control of proteolysis during development and/or germination, and (3) protection of seeds again pest (Barret 1987). In the case of taro, cystatin is unique in Kaohsiung No. 1 cultivar, and spatially expressed in corm tissue. We thereafter propose that the most excellent pest-resistance of Kaohsiung No. 1 in the local taro cultivars is highly correlated with the cystatin gene function.

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－編輯：謝兆樞