Canadian Journal of Plant Science
Revue canadienne de phytotechnie

CsPDC-E1 α , a novel pyruvate dehydrogenase complex E1 α subunit gene from *Camellia sinensis*, is induced during cadmium inhibiting pollen tube growth

Journal:	<i>Canadian Journal of Plant Science</i>
Manuscript ID	CJPS-2017-0074.R2
Manuscript Type:	Article
Date Submitted by the Author:	31-May-2017
Complete List of Authors:	Pan, Juntong; Nanjing Agricultural University, College of Horticulture Li, Dongqin Shu, Zaifa Jiang, Xin Ma, Wenwen Wang, Weidong Zhu, Jiaojiao Wang, Yuhua; Nanjing Agricultural University, College of Horticulture
Keywords:	CsPDC-E1 α , <i>Camellia sinensis</i> , pollen tube, Cd stress, expression analysis

SCHOLARONE™
Manuscripts

CsPDC-E1 α , a novel pyruvate dehydrogenase complex E1 α subunit gene from *Camellia sinensis*, is induced during cadmium inhibiting pollen tube growth

Junting Pan^{1†}, Dongqin Li^{1†}, Zaifa Shu¹, Xin Jiang^{1,3}, Wenwen Ma², Weidong Wang^{1,4}, Jiaojiao Zhu¹, Yuhua Wang^{1*}

¹College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

²Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China

³Linhai Specialty and Technology Extension Station, Linhai 317000, Zhejiang, China

⁴College of Horticulture, Northwest A&F University, Yangling 712100, Shaanxi, China

Author E-mail

Junting Pan: 2014104092@njau.edu.cn

Dongqin Li: 2014104093@njau.edu.cn

Zaifa Shu: 2013104093@njau.edu.cn

Xin Jiang: jiang0xin@126.com

Wenwen Ma: mawenwen@sinap.ac.cn

Weidong Wang: wangweidong@nwafu.edu.cn

Jiaojiao Zhu: 2016104092@njau.edu.cn

[†]These authors contributed equally to this work.

*Correspondence:

Yuhua Wang

College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

Tel: +86-25-84395182

Fax: +86-25-84395182

E-mail: wangyuhua@njau.edu.cn

Abstract: Cadmium (Cd) is one of the most toxic heavy metal pollutants highly hazardous to pollen tubes by disrupting mitochondria. Mitochondrial pyruvate dehydrogenase complex (PDC) plays important roles in cellular metabolism and links cytosolic glycolytic metabolism to the tricarboxylic acid cycle (TCA). However, the relationship between PDC and pollen germination and pollen tube growth under Cd stress remains unclear. Here we found that Cd inhibited *Camellia sinensis* pollen germination and pollen tube growth in a dose dependent manner and disrupted the tip clear zone of pollen tube. Furthermore, we isolated a novel PDC gene (*CsPDC-E1 α*) from *C. sinensis*. The full-length cDNA of *CsPDC-E1 α* was 1606 bp and encoded a 393-amino acid protein containing typical PDC TPP-binding site, heterodimer interface, phosphorylation loop region and tetramer interface domain, suggesting that *CsPDC-E1 α* was a member of the PDC_ADC_BCADC subfamily in thiamine pyrophosphate (TPP) family. Bioinformatics analysis indicated that the *CsPDC-E1 α* protein shared high degree of homology with that from *Petunia x hybrid*. The *CsPDC-E1 α* relative expression levels in pollen were significantly higher than other tissues of *C. sinensis*, indicating that *CsPDC-E1 α* expression is tissue-specific. To confirm the functions of *CsPDC-E1 α* in pollen response to Cd stress, we analyzed the relative expression level of *CsPDC-E1 α* in Cd-treated pollen tubes and found that the expression of *CsPDC-E1 α* was induced by Cd stress. All these results indicate that *CsPDC-E1 α* might be associated with Cd inhibition of *C. sinensis* pollen germination and pollen tube growth.

Keywords: *CsPDC-E1 α* , *Camellia sinensis*, pollen tube, Cd stress, expression analysis

Introduction

Heavy metals contaminations in soil are the main factor negatively affecting crop growth and productivity. Among them, Cadmium (Cd) is one of the most toxic trace heavy metals and has become a major environmental pollutant. Cd can be

accumulated in all plant parts and affect many vital processes, which causes stunted growth, chlorosis, leaf epinasty, alters the chloroplast ultrastructure, inhibits photosynthesis, inactivates enzymes in CO₂ fixation, induces lipid peroxidation and also disturbs the nitrogen (N) and sulfur (S) metabolism and antioxidant machinery (Perfus-Barbeoch et al. 2002; Khan et al. 2007; Mobin and Khan, 2007; Iqbal et al. 2010; Marquez-Garcia et al. 2011). In some plant species, Cd toxicity is manifested at cellular level as chromosomal aberrations and alteration of cell cycle and division (Gallego et al. 2012). High mutation rate and malformed embryos have been observed in *Arabidopsis* exposed to Cd (Ernst et al. 2008). In terms of *C. sinensis*, a few studies have shown that Cd exposure induced stress in buds of tea plants and up-regulated the transcription of glutathione and glutamine metabolic genes (Mohanpuria et al. 2007; Rana et al. 2008). To our knowledge, there are few observations of the effects of Cd stress on other tissues of tea plants. Hence, the effects of Cd pollution on tea plants deserve more attention.

Pollen is considered to be more sensitive to pollutants than vegetative parts of the plants (Mulcahy 1981). Pollen germination and tube growth are inhibited in the presence of various heavy metals, especially Cd. Previous reports indicated that Cd strongly inhibited pollen germination and tube growth (Xiong and Peng 2001; Sawidis 2008; Sabrine et al. 2010). Increased Cd concentrations completely paralyzed cytoplasmic streaming and relocated cell organelles (plastids, lipid droplets) in *Lilium longiflorum* and *Nicotiana tabacum* pollen tube tips (Sawidis 2008). The size of apical zone of pollen tube was also drastically reduced, however, the diameter of the entire tip increased. The distribution of vesicles into the subapical region was altered and mitochondria became dispersed throughout the subapical region. Furthermore, a previous study in *Picea wilsonii* revealed that Cd stress strongly inhibited pollen germination and tube growth by inhibiting endo/exocytosis, inducing the formation of acidic vacuoles, resulting in swollen tube tips and irregularly broadened tube diameters (Wang et al. 2014). Current information is insufficient to determine the mechanism by which Cd to inhibit pollen germination and tube growth.

The pyruvate dehydrogenase complex (PDC) represents a member of a multi-enzyme complex family of the 2-oxoglutarate dehydrogenase complex (OGDHC) and the branched-chain 2-oxoacid dehydrogenase complex (BCOADC). The PDC catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA and NADH and is composed of three fundamental enzymatic components: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) (Reid et al. 1977). E1 is composed of alpha (E1 α) and beta (E1 β) subunits (de Kok et al. 1998). The mammalian and yeast mitochondrial PDC (mtPDC) consists of 20–30 E1 heterotetramers (E1 α and E1 β subunits), one E2 60mers (20 trimers), and 12 E3 dimers (Reed 2001). In this large multi-enzyme assembly, E2 forms the core structure to which E1 α 2 β 2 heterotetramers and E3 homodimers attach. Previous experiments showed that plant mtPDC activity is, in fact, regulated by product inhibition, metabolites, and reversible phosphorylation (Luethy et al. 1996; Budde et al. 1991) like mammalian mtPDC (Patel and Roche 1990). The E1 α protein levels were highest in maize pollen, which was at least 2-fold higher than any other examined organs (Thelen et al. 1999). The relative abundance of E1 α protein in nonphotosynthetic tissues may reflect a high cellular content of mitochondria, a high level of respiratory activity, or an extra plastidial requirement for acetate (Thelen et al. 1999). However, there is no evidence about Cd stress affecting pollen germination and growth by affecting the E1 α . Thus, the underlying basis of the PDC mechanisms of pollen tube growth under Cd stress needs more research.

Here, we investigated the *C. sinensis* pollen germination and tube elongation in response to Cd stress. In addition, we isolated a novel *PDC-E1 α* gene from *C. sinensis* (*CsPDC-E1 α*), and investigated the bioinformatics analysis of this putative *CsPDC-E1 α* gene. Furthermore, the organ-specific expression of this gene was analyzed, and the expression levels of *CsPDC-E1 α* in response to Cd stress were monitored to explore the role of this gene during Cd stress.

Materials and Methods

Plant material and in vitro pollen culture

Mature pollen grains were collected from *C. sinensis* (L.) O. Kuntze cv. 'Longjing43' tea plants. For all experiments, pollen grains were incubated in pollen germination solution (containing 30 mM MES, 5% (w/v) sucrose, 0.01% (w/v) H_3BO_3 , 0.05% (w/v) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 5% (w/v) PEG 4000, pH 6.0) at 25 °C in the dark in vitro. To examine the effect of Cd on pollen germination and tube growth, pollen grains from *C. sinensis* were incubated for 20, 40, 80 or 120 min in germination solution containing different concentrations of CdCl_2 (0, 100, 200 and 400 μM). Mean germination rate was determined for each treatment Cd concentration after 120 min in three replicates. Mean tube length and germination rate for at least 50 pollen tubes was determined in each of three replicates at 20, 40, 80 and 120 min exposure to each Cd concentration. The morphology of the pollen tubes was examined using a Leica DM2500 biological microscope, and digital images were captured with a Leica DFC290 digital color camera (Leica, Germany).

RNA extraction and cDNA synthesis

The roots, stems, leaves, buds, flowers, fruits and pollen grains were harvested separately from tea plants for qRT-PCR analysis of *CsPDC-E1 α* gene. The tissues were washed with deionized water, weighed, and stored at -80°C until use. A *CsPDC-E1 α* cDNA was clone from pollen grain RNA. Expression analysis of *CsPDC-E1 α* gene was observed in pollen grain germinated for 40, 80 and 120 min in the germination solution containing 0, 200 or 400 μM Cd. Total RNA was extracted from these plant samples using RNAiso Plus (TaKaRa, Japan) according to the manufacturer's protocol, and the RNA was treated with RNase-free DNaseI (TaKaRa, Japan) to remove potential genomic DNA contamination. The quality of the total RNA was measured using a ONE DropTM OD-1000+ spectrophotometer (ONE Drop, USA). The first-strand cDNA was synthesized using the Prime ScriptTM 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions.

Cloning and sequence analysis

To acquire the internal conserved fragment from closing to the N-terminal, specific primer and degenerate primer (CsPDC-E1 α -5'GSP and CsPDC-E1 α -5'JB) were designed and synthesized according to the conserved amino acids of PDC-E1 α proteins from *Arabidopsis*, *Vitis vinifera*, *Brassica rapa* and *Zea mays*. Then, the conserved sequence of CsPDC-E1 α was amplified using PCR from *C. sinensis* cDNA. Subsequently, the 5' and 3'UTR sequences were identified by RACE using the specific primers CsPDC-E1 α -5'GSP1/5'GSP2 and CsPDC-E1 α -3'GSP1/3'GSP2. Finally, a pair of gene-specific primers (CsPDC-E1 α -Full-F and CsPDC-E1 α -Full-R) was designed and used to amplify the full-length sequence of CsPDC-E1 α , the product was then cloned into the pMD[®]18-T Vector (TaKaRa, Dalian, China) for sequencing (GenScript, China). The primers used in cloning of CsPDC-E1 α were listed in Table 1.

DNAMAN software version 5.2.2 (Lynnon Biosoft, St Louis, QC, Canada) and BLAST online (Coordinators 2017) were used for alignment of the DNA and protein sequences. Phylogenetic tree was conducted using MEGA 5.05. The amino acid basic components of CsPDC-E1 α protein was analyzed by ProtParam tool (<http://www.expasy.ch/tools/protparam.html>). The secondary structure of CsPDC-E1 α protein was predicted using Predictprotein software (<https://www.predictprotein.org/>). The online tools WoLF PSORT (<https://wolfpsort.hgc.jp/>) and Softberry ProComp v9.0 (<http://www.softberry.com/>) were used to predict CsPDC-E1 α protein localization.

Quantitative real-time PCR (qRT-PCR)

QRT-PCR assays were performed using SYBR[®] Premix Ex Taq[™] (TaKaRa, Japan) and an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) according to the manufacturer's instructions. The specific primers CsPDC-E1 α -qF and CsPDC-E1 α -qR were used to amplify a 142-bp CsPDC-E1 α fragment. Cs β -Actin was selected as an internal control. The primers used in the qRT-PCR are listed in Table 1.

The qRT-PCR conditions were as follows: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 59 °C for 30 s. Relative gene expression levels were estimated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

For each biological replicate, three technical repeats were performed. And all data were represented as the means $\pm$ standard deviations (SD). Group differences were tested using one-way ANOVA and Duncan's test, and significant differences were represented by different letters ($P < 0.05$). Data analysis was performed using SPSS 20 software.

Results

Sequence analysis of *CsPDC-E1 α*

The full length of *CsPDC-E1 α* (NCBI GenBank No. JQ99982) is 1606 bp with an 1182 bp ORF, 105 bp 5'UTR and 319 bp 3'UTR (Fig. 1). There is a conserved AATAAA sequence after the terminator TGA, which provides signal for chain-cutting and polyadenylation. Typical Poly (A) lies in the end of the *CsPDC-E1 α* cDNA. The predicted protein of *CsPDC-E1 α* has 393 amino acid residues. According to ProtParam analysis, the coding product belongs to stable protein with the calculated isoelectric point (pI) 7.59, the formula is $C_{1927}H_{3022}N_{534}O_{573}S_{25}$ and the molecular weight is 43.64 kDa.

The putative protein coded by *CsPDC-E1 α* contains two conserved motifs, thiamine pyrophosphate (TPP) binding site and PDC β -Binding site (Fig. 2). The *CsPDC-E1 α* protein showed 84.46% similarity compared to other species of proteins and the homologous alignment shows that the amino acid sequence of N-terminal of *CsPDC-E1 α* (1-60 bp, 52.19% identity) is less conserved than the C-terminal (333-393 bp, 85.83% identity). Three serine phosphorylation sites found in mammalian PDC-E1 α are also identified in *CsPDC-E1 α* .

The phylogenetic relationship of PDCE1 α protein from different plant species was determined by cluster analysis (Fig 3). *CsPDC-E1 α* is more similar to PhPDCE1 α (*Petunia x hybrida*), VvPDCE1 α (*Vitis vinifera*) and GhPDCE1 α (*Gossypium*

hirsutum) than to PDC-E1 α from other dicots and has an even greater difference to PDC-E1 α from monocots (ZmPDCE1 α (*Zea mays*) and OsPDCE1 α (*Oryza sativa* Japonica Group)).

Conserved domain prediction by protein BLAST of NCBI shows that CsPDC-E1 α belongs to the PDC_ADC_BCADC subfamily of the thiamine pyrophosphate family (Supplementary Fig. S1). The TPP-binding site, heterodimer interface, phosphorylation loop region and tetramer interface are located between amino acid residues 33 and 393. The subcellular localization prediction using WoLF PSORT and Softberry ProComp v9.0 shows that CsPDC-E1 α protein locates to the mitochondria. The secondary structure of CsPDC-E1 α , predicted by Predictprotein software, consists of 37.24% α -helix, 18.11% sheet, 9.18% β -turn and 35.46 % random coil (Supplementary Fig. S2).

***CsPDC-E1 α* expression analysis in tea plant tissues**

To determine the expression pattern of *CsPDC-E1 α* in the different tissues of tea plant, qRT-PCR analysis of *CsPDC-E1 α* was performed using the total RNA extracted from roots, stems, leaves, shoots, pollen grains and fruits. A relatively low expression level of *CsPDC-E1 α* was detected in roots, stems, leaves, shoots and fruits (Fig. 4A), while the expression of *CsPDC-E1 α* in pollen grains was significantly higher than that in other tissues ($P < 0.05$). Further expression analysis of different floral organs was performed and showed higher *CsPDC-E1 α* expression levels in petal and receptacle ($P < 0.05$) than in calyx, stamen and stigma (Fig. 4B). Lower levels of *CsPDC-E1 α* were expressed in stamens, but a significantly higher level was found in pollen grains compared to non-reproductive tissues (Fig. 4A and 4B). This indicates that the expression of *CsPDC-E1 α* is tissue or organ specific.

Effects of Cd on pollen germination, pollen tube elongation and morphology

Microscopy was used to ascertain whether Cd affected pollen tube development, pollen germination, pollen tube elongation and morphology. Compared to the control

(Fig. 5A), decreased pollen germination was observed in pollen grains treated with 100, 200 and 400 μM Cd after 20, 40, 80 and 120 min. Cd also caused a strong decrease in pollen tube elongation in comparison to controls with increasing of Cd concentration (Fig. 5B). These results demonstrate that Cd significantly reduces pollen germination and pollen tube elongation in a dose and exposure dependent manner.

Additionally, Cd induced morphological changes of pollen tubes, particularly in the apical and sub-apical regions. When exposed to different concentrations of Cd, pollen tubes showed a range of morphological abnormalities, characterized by swollen tips of the pollen tube and irregularly broadened tube diameters (Fig. 6).

Expression patterns of *CsPDC-E1 α* in response to Cd stress

Tea pollen tubes were exposed to 200 μM and 400 μM Cd to study the expression patterns of *CsPDC-E1 α* over a time course during Cd exposure. As shown in Fig. 7, the expression of the gene increased significantly after Cd stress, especially at 200 μM for 120 min and 400 μM for 40 min. The relative expression of *CsPDC-E1 α* under Cd stress is mostly higher than the control (0 μM).

Discussion

Plants have distinct, spatially separated types of PDCs in mitochondria and plastids (Rahmatullah et al. 1989). The plastid form PDCs provide acetyl-CoA and NADH for fatty acid biosynthesis (Lernmark and Gardestrom 1994), whereas the mitochondria form PDCs are important in controlling the entry of carbon into the TCA cycle (Camp and Randall 1985). Most mitochondrial and plastidial proteins, including the subunits of the PDCs, are encoded within the nuclear genome of land plants, synthesized in the cytoplasm and then post-translationally imported into the organelles (Mooney et al. 2002). In the present study, *CsPDC-E1 α* was isolated from *C. sinensis* and phylogenetic analysis of plant PDC-E1 α proteins revealed that *CsPDC-E1 α* shares a high degree of homology with *PhPDC-E1 α* (*Petunia x hybrida*) and *VvPDC-E1 α*

(*Vitis vinifera*) (Fig. 3). Our previous study showed that CsPDC-E1 α located to the mitochondria (Du et al. 2015). All these results confirm that *CsPDC-E1 α* belongs to mtPDC and might function in the mitochondria. Pollen germination occurs rapidly during pollination, and is critical for successful sexual reproduction in plants. A series of cellular components are formed and a large number of genes are coordinately expressed in the pollen germination and growth process (Wang et al. 2012). This increased amount of cellular activity results in an increased demand for mitochondrial respiration and TCA cycle-derived metabolic products. Previous studies have noted that the expression of plant mtPDC was high in anther (Thelen et al. 1999; Luethy et al. 2001). Furthermore, antisense inhibition of PDC-E1 α subunit in anther tapetum causes male sterility (Yui et al. 2003). In addition, Thelen et al. (1999) reported that one E1 α subunit and three E1 β subunits were isolated from maize and their expression levels were the highest in pollen. In our study, the pollen *CsPDC-E1 α* relative expression levels were significantly higher in pollen grains than the other tissues (Fig. 4). The results indicate that *CsPDC-E1 α* expression is tissue-specific and suggests it plays an important role in *C. sinensis* pollen germination and pollen tube growth.

Cd is a toxic metal with a long biological half-life which can be easily absorbed by plants. High Cd concentrations disrupt control of the cell physiological state and trigger a number of complex physiological changes. We have shown here that Cd reduces pollen germination and alters tube growth (Sawidis 2008; Sabrine et al. 2010). Others, Xiong et al. (2001) for example, reported that Cd might adversely affect *Vicia angustifolia* and *Vicia tetrasperma* reproduction by inhibiting either pollen germination or tube growth even at 0.01 $\mu\text{g/mL}$ concentrations. Furthermore, Cd not only strongly prevented *Picea wilsonii* pollen germination and tube growth in a dose-dependent manner, but also caused significant morphological alterations, including cytoplasmic vacuolization, swollen tips and irregular tube diameters (Wang et al. 2014). Our data (Fig. 6) is consistent with these previous reports. Additionally, in *Arabidopsis* root tip mitochondria, exposure to high concentration of Cd caused swelling and altered morphology, degradation of internal membranes and loss of

cristae structure (Fan et al. 2011). More importantly, the mitochondria were distributed in *Lilium longiflorum* and *Nicotiana tabacum* pollen tube tip throughout the subapical region and mixed with the vesicles of the tube apex under increased Cd concentrations (Sawidis 2008).

Here, we found that the relative expression of *CsPDC-E1 α* increased over time in pollen tubes exposed to 200 μ M Cd. Combining a previous report (Du et al. 2015) and our findings, we therefore speculate that the increased expression of *CsPDC-E1 α* in pollen grains induced by Cd stress results in the disruption of energy metabolism and eventually leads to the irregular growth of *C. sinensis* pollen tubes. Additionally, our results showed that pollen tubes treated by high concentration of Cd (400 μ M) were disrupted drastically, especially after the longest treatment (120 min) (Fig. 5 and Fig. 6). According to previous reports, pollen grains cultured in Cd showed a range of morphological abnormalities, had slower pollen tube slow growth and eventually died (Sawidis 2008). We speculate that the expression of *CsPDC-E1 α* is decreases with increasing exposure to 400 μ M Cd due to a severe disruption of vitality and reduced transcriptional competence. Despite this, the relative expression of *CsPDC-E1 α* under Cd stress remains higher than the control (Fig. 7), indicating that *CsPDC-E1 α* is induced by Cd stress.

In summary, a *CsPDC-E1 α* gene was isolated from *C. sinensis*, and it shares a high degree of homology with *PhPDC-E1 α* . The expression of *CsPDC-E1 α* is tissue-specific and shows the highest expression level in pollen grains. Furthermore, the *CsPDC-E1 α* is induced by Cd exposure. Increased exposure of *C. sinensis* pollen to Cd reduces pollen germination and pollen tube growth and disrupts pollen tube morphology compared to controls. All these results indicate that *CsPDC-E1 α* might be associated with Cd inhibition of *C. sinensis* pollen germination and pollen tube growth.

Acknowledgements

This work was supported by the Fundamental Research Funds For the Central

Universities (No. KYZ201509), the National Natural Science Foundation of China (No. 31370014) and the Opening Project of State Key Laboratory of Tea Plant Biology and Utilization (No. SKLTOF20150113).

References

- Budde, R. J. A., Fang, T. K., Randall, D. D. & Meeenyk, J. A. 1991. Acetyl-coenzyme-a can regulate activity of the mitochondrial pyruvate-dehydrogenase complex insitu. *Plant Physiology*, **95**, 131-136.
- Camp, P. J. & Randall, D. D. 1985. Purification and characterization of the pea chloroplast pyruvate-dehydrogenase complex - a source of acetyl-coa and nadh for fatty-acid biosynthesis. *Plant Physiology*, **77**, 571-577.
- Coordinators, N. R. 2017. Database Resources of the National Center for Biotechnology Information. *Nucleic Acids Res*, **45**, D12-D17.
- De Kok, A., Hengeveld, A. F., Martin, A. & Westphal, A. H. 1998. The pyruvate dehydrogenase multi-enzyme complex from Gram-negative bacteria. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology*, **1385**, 353-366.
- Du, Y. L., Wang, W. D., Wang, Y. H., LI, X. H. 2015. Subcellular localization of CsE1 α as well as cloning and expression of its promoter from the pollen of *Camellia sinensis*. *Journal of Tea Science*, **3**, 290-298.
- Ernst, W. H. O., Krauss, G. J., Verkleij, J. A. C. & Wesenberg, D. 2008. Interaction of heavy metals with the sulphur metabolism in angiosperms from an ecological point of view. *Plant Cell and Environment*, **31**, 123-143.
- Fan, J. L., Wei, X. Z., Wan, L. C., Zhang, L. Y., Zhao, X. Q., Liu, W. Z., Hao, H. Q. & Zhang, H. Y. 2011. Disarrangement of actin filaments and Ca²⁺ gradient by CdCl₂ alters cell wall construction in *Arabidopsis thaliana* root hairs by inhibiting vesicular trafficking. *Journal of Plant Physiology*, **168**, 1157-1167.
- Gallego, S. M., Pena, L. B., Barcia, R. A., Azpilicueta, C. E., Lannone, M. F., Rosales, E. P., Zawoznik, M. S., Groppa, M. D. & Benavides, M. P. 2012. Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environmental and Experimental Botany*, **83**, 33-46.
- Iqbal, N., Masood, A., Nazar, R., Syeed, S. & Khan, N. A. 2010. Photosynthesis, growth and antioxidant metabolism in mustard (*Brassica juncea* L.) cultivars differing in cadmium tolerance. *Agricultural Sciences in China*, **9**, 519-527.
- Khan, N. A., Singh, S. S. & Nazar, R. 2007. Activities of antioxidative enzymes, sulphur assimilation, photosynthetic activity and growth of wheat (*Triticum aestivum*) cultivars differing in yield potential under cadmium stress. *Journal of Agronomy and Crop Science*, **193**, 435-444.
- Lernmark, U. & Gardestrom, P. 1994. Distribution of pyruvate-dehydrogenase complex activities between chloroplasts and mitochondria from leaves of different species. *Plant Physiology*, **106**, 1633-1638.

- Livak, K. J. & Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods*, **25**, 402-408.
- Luethy, M. H., Gemel, J., Johnston, M. L., Mooney, B. P., Miernyk, J. A. & Randall, D. D. 2001. Developmental expression of the mitochondrial pyruvate dehydrogenase complex in pea (*Pisum sativum*) seedlings. *Physiologia Plantarum*, **112**, 559-566.
- Luethy, M. H., Miernyk, J. A. & Randall, D. D. 1996. Developmental expression of the mitochondrial pyruvate dehydrogenase complex. *Plant Physiology*, **111**, 83-83.
- Marquez-Garcia, B., Horemans, N., Cuypers, A., Guisez, Y. & Cordoba, F. 2011. Antioxidants in *Erica andevalensis*: A comparative study between wild plants and cadmium-exposed plants under controlled conditions. *Plant Physiology and Biochemistry*, **49**, 110-115.
- Mobin, M. & Khan, N. A. 2007. Photosynthetic activity, pigment composition and antioxidative response of two mustard (*Brassica juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress. *Journal of Plant Physiology*, **164**, 601-610.
- Mohanpuria, P., Rana, N. K. & Yadav, S. K. 2007. Cadmium induced oxidative stress influence on glutathione metabolic genes of *Camellia sinensis* (L.) O. Kuntze. *Environmental Toxicology*, **22**, 368-374.
- Mooney, B. P., Miernyk, J. A. & Randall, D. D. 2002. The complex fate of alpha-ketoacids. *Annual Review of Plant Biology*, **53**, 357-375.
- Mulcahy, D. L. 1981. Pollen tetrads in the detection of environmental mutagenesis. *Environ Health Perspect*, **37**, 91-94.
- Patel, M. S. & Roche, T. E. 1990. Molecular-biology and biochemistry of pyruvate-dehydrogenase complexes. *Faseb Journal*, **4**, 3224-3233.
- Perfus-Barbeoch, L., Leonhardt, N., Vavasseur, A. & Forestier, C. 2002. Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *Plant Journal*, **32**, 539-548.
- Rahmatullah, M., Gopalakrishnan, S., Radke, G. A. & Roche, T. E. 1989. Domain-structures of the dihydrolipoyl transacetylase and the protein-X components of mammalian pyruvate-dehydrogenase complex-selective cleavage by protease arg C. *Journal of Biological Chemistry*, **264**, 1245-1251.
- Rana, N. K., Mohanpuria, P. & Yadav, S. K. 2008. Expression of tea cytosolic glutamine synthetase is tissue specific and induced by cadmium and salt stress. *Biologia Plantarum*, **52**, 361-364.
- Reed, L. J. 2001. A trail of research from lipoic acid to alpha-keto acid dehydrogenase complexes. *Journal of Biological Chemistry*, **276**, 38329-38336.
- Reid, E. E., Thompson, P., Lyttle, C. R. & Dennis, D. T. 1977. Pyruvate dehydrogenase complex from higher plant mitochondria and proplastids. *Plant Physiol*, **59**, 842-848.
- Sabrine, H., Afif, H., Mohamed, B., Hamadi, B. & Maria, H. 2010. Effects of cadmium and copper on pollen germination and fruit set in pea (*Pisum sativum*

- L.). *Scientia Horticulturae*, **125**, 551-555.
- Sawidis, T. 2008. Effect of cadmium on pollen germination and tube growth in *Lilium longiflorum* and *Nicotiana tabacum*. *Protoplasma*, **233**, 95-106.
- Thelen, J. J., Miernyk, J. A. & Randall, D. D. 1999. Molecular cloning and expression analysis of the mitochondrial pyruvate dehydrogenase from maize. *Plant Physiol*, **119**, 635-644.
- Wang, W. Y., Zhang, L., Xing, S. F., Ma, Z. Q., Liu, J. J., Gu, H. Y., Qin, G. J. & Qu, L. J. 2012. *Arabidopsis AtVPS15* Plays essential roles in pollen germination possibly by interacting with *AtVPS34*. *Journal of Genetics and Genomics*, **39**, 81-92.
- Wang, X. X., Gao, Y., Feng, Y., Li, X., Wei, Q. & Sheng, X. Y. 2014. Cadmium stress disrupts the endomembrane organelles and endocytosis during *Picea wilsonii* pollen germination and tube growth. *Plos One*, **9**.
- Xiong, Z. T. & Peng, Y. H. 2001. Response of pollen germination and tube growth to cadmium with special reference to low concentration exposure. *Ecotoxicology and Environmental Safety*, **48**, 51-55.
- Yui, R., Iketani, S., Mikami, T. & Kubo, T. 2003. Antisense inhibition of mitochondrial pyruvate dehydrogenase E1 alpha subunit in anther tapetum causes male sterility. *Plant Journal*, **34**, 57-66.

Fig. 1. Nucleotide and deduced amino acid sequences of *CsPDC-E1 α* cDNA. The full length of *CsPDC-E1 α* is 1606 bp with a 1182 bp ORF, including 105 bp 5'UTR and 319 bp 3'UTR.

Fig. 2. Alignment of *CsPDC-E1 α* amino acid sequence with other plant PDCE1 α proteins. Black and grey show 100% and 50%–99% identity on amino sequences, respectively. The three residues phosphorylated on the mammalian PDCE1 α subunit are indicated by asterisk.

Fig. 3. Phylogenetic analysis of *CsPDC-E1 α* proteins with other plants PDCE1 α proteins. Cluster analysis of amino acid sequence of PDC-E1 α in different plant species conveys the phylogenetic relationship information. A phylogenetic tree was constructed using *MEGA 5.05* and the neighbor-joining method.

Fig. 4. Expression patterns of *CsPDC-E1 α* in different tissues. *Cs β -Actin* was used as internal control. The data represent the means of three replicates. A: *CsPDC-E1 α* expression in different tissues of tea plant; B: *CsPDC-E1 α* expression at different part of tea flower.

Fig. 5. Effects of Cd stress on *C. sinensis* pollen germination and tube growth. A: pollen germination rate at different time under different Cd concentration treatment. B: pollen tube average length in different time under different Cd concentration treatment.

Fig. 6. Changes in morphology of *C. sinensis* pollen tubes after treatment with different Cd concentrations for 120 minutes. A: 0 μ M Cd treatment; B: 200 μ M Cd treatment; C: 400 μ M Cd treatment.

Fig. 7. Expression patterns of *CsPDC-E1 α* in response to Cd stress. The expression of

the gene was increased significantly at 40 min as soon as expose to Cd stress, especially at 200 μ M. After 80 min or 120 min exposure, the relative expression of *CsPDC-El α* remains higher levels after treated with Cd stress than the control (0 μ M).

For Review Only

Table 1. Primers used in this study (R = A/G, Y = C/T, N = A/C/T/G, I = A/C/T/G).

Primer name	Sequence(5'-3')	Description
AP	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTT	
5'AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	
AUAP	GGCCACGCGTCGACTAGTAC	
CsPDC-E1 α -3'GSP1	TGGTATGGATGCCTTTGCTGTGA	3'RACE
CsPDC-E1 α -3'GSP2	ATCGCCACCGAAGAGGAACTGAA	
CsPDC-E1 α -5'GSP	TTCAGTTCCTCTTCGGTGGC	5'RT-PCR
CsPDC-E1 α -5'JB	ATGGCKYTGATGCGNCGNATGGA	
CsPDC-E1 α -5'GSP1	TGTTTCACAGCAAAGGCATC	5'RACE
CsPDC-E1 α -5'GSP2	TCCGCAAACGCCTCAAGTAG	
CsPDC-E1 α -Full-F	ACATACCAAACCCTTCAACT	RT-PCR
CsPDC-E1 α -Full-R	GAAATAAAGCCGAAAATCAA	
CsPDC-E1 α -qF	CGGTTTCTGCCACCTCTACGACG	qRT-PCR
CsPDC-E1 α -qR	AAACGCCTCAAGTAGGGTTCCGC	
Cs β -actin-qF	GCCATCTTTGATTGGAATGG	
Cs β -actin-qR	GGTGCCACAACCTTGATCTT	

TTCTCTCTCTGCTCACATACCAACCCCTCAACTTCTCAGGCTCCATTTTCAGAGAGAGAAAACGAGCAGAGATCAGAGAAAAGTCAGGCAACGAACGATCAAG
ATGGCTCTGACTCACCCTGATTCGAATCTGCTGAAGCCGTTCTCACCCTGCTCTACACGCCATCTCTCCGCGGTTCAATTTCCACGGACGATTAACCTCCATCACCCTCGAGAGC
M A L T H R Y S N L L K P F S T A V S Y T P S L R R S F S T D D S T S I T V E T
AGTGTCCCTTCACGGCCACAACTGCGAACCAACCTGCTGCAACCTCACCACCCCAAGAGCTCATAACCTTCTCCGAGATATGGCGATGATGCGTGGATGGAGATCGCCGCC
S V P F T G H N C E P P S R T V T T T P K E L I T F F R D M A M M R R M E I A A
GATTCGCTCTACAAAGCGAAGCTGATCCGCGGTTTCTGCCACCTCTACGACGCCAAGAAGCGGTGGCGATCGGAATGGAAGCGGCTATCACCAGAAAGGATTGCATCATCACCCTAT
D S L Y K A K L I R G F C H L Y D G Q E A V A I G M E A A I T K K D C I I T A Y
CGCGACCTGCTCTCTCCGCGCGCGGGAACCTACTTGAGGCGTTTGGGAGCTGATGGGACGAAAAGCTGGGTGTTGGAAGGAAAAGGTGGTTCATGCATTTTACAAGAAG
R D H C L F L G R G G T L L E A F A E L M G R K A G C S K G K G S M H F Y K K
GAAAGTGATTCATGGAGGTCATGGGATTGTTGGGCTCAAGTGCCTTGAAGTGTGGATGGCTTGTCTCAGAACTACTCAGGAAGAGCATGTGACCTTACAATGTATGCTGAT
E S G F Y G G H G I V G A Q V P L G C G L A F A Q K Y S K E E H V T F T M Y G D
GGTGTGCGAATCAGGACAGTTGTTGAGGCGCTTAATATGGCGCGCTTGGGATCTCCCTGCGATTTTGGTTTGGGAAAACATCACTATGGAATGGGACAGCTGAATGGAGAGCC
G A A N Q G Q L F E A L N M A A L W D L P A I L V C E N N H Y G M G T A E W R A
GCTAAGAGTCTGCTTATTAAGAGAGGCGATTATGTTCTGGCTTGAAGGTAGATGGATGGATGCTTGTGTGAAACAACTTGCAAAATTTGCCAAGGAGCACGCTTGAAGAAT
A K S P A Y V Y K R G D V V P G L K V D G M D A F A V K Q A C K F A K E H A L K N
GGACCCATTATCTTGAATGGACACTTACAGGTACCATGGTCACTCCATGCTGATCCTGGGAGTACCTATCGCACAGTGATGAAATTAAGTGTGAGACAGGAGCGTGATCCAATT
G P I I L E M D T Y R Y H G H S M S D P G S T Y R T R D E I T G V R Q E R D P I
GAAAGAATAAGAAAGCTGGTACTATCTCATGATATCGCCACCGAAAAGGAAGTGAAGGATATGGAGAAGGAAATCAGAAAAGAGGTAGATGAAGCCATCGCTCAAGCCAGGAAAATCCCT
E R I R K L V L S H D I A T E K E L K D M E K E I R K E V D E A I A Q A R E I P
ATGCCAGATCTTCTGAACCTTACCAACGTGTATGTTAAAGGCTTGGAGTTGAGGATCGCGCGCAGATAGGAAAGAGCCAGGCGCTGCTTCTTGA
M P D P S E L F T N V Y V K G F G V E A C G A D R K E A R A V L P *
GAAATTTCAACACTCGCCTTTTGGACCAATAA GAAATTAATGTGAAATTTTGAATAAAAGCTGCACCATCTGCTATGGGCTGTGGAGTTGGGCTGTTCTTTTCTTTCTC
TGGGTATGGAAAAATTTATTTCTGTTATTTGGCTAGAAAAATTTAGCTATTTTGTATTTGCTGCTAAATTTGAATATGCTTCCCAAGTGCTTAATGGAGTTCCAGATTAICTGTTGT
AAATTGATTTTCGGCTTATTTTCATCAATGTTTATGATTTAGTTTCATGTTCTCTTGAAAAAATTTTTTTTTT

70x33mm (300 x 300 DPI)

CsPDCE1a	.NALTHR.....YSNLLKPF..TAVSYIPSLRSEETDDST..SITVETSVETGNCDEPESSTVT
PhPDCE1a	.NALSTTRT....TVSRFLAFLT..TAVSIT...RHLSTIN....LTIDTETSLPFTGNAIDPSSSTVE
VvPDCE1a	.NALSTS.....FRSNLIKPLTALTALSSIQHLRPFSSADDSTVLTIDTETSVETGNCDEPESRAVE
GhPDCE1a	.NALSHLAASA..SRSSSLKPLS..SALSRL....RPISSGNS...PLTIVETSVETGNCDEPESRAVE
BrPDCE1a	.NALSRLSSRS.....NIISRPLS..AAFR.....RPISTDTTP...ITIDTETSLPFTALCDPESRSVE
AtPDCE1a	.NALSRLSSRS.....NIISRPLS..AAFR.....RPISTDTTP...ITIDTETSLPFTALCDPESRSVE
GmPDCE1a	.NALSRLSHRSIGSTLTKEFFSAAA..ASAASS....RPISTSDTP...LTIDTETSVETGNCDEPESRAVA
PsPDCE1a	.NALSRLSSSSSSNGSNLNFPS..AAFTLN...RPISSDTTAT..LTIDTETSLPFTALCDPESRSVT
OsPDCE1a	MAFAVLLRLRLG..VIAAFRRAA..AALFLT..TSVGVGSDTEF...LTIDTETSVETGNCDEPESRAVA
ZmPDCE1a	.KFAAALLRRIF.....AARAPFA..TAF....MAAFVGDSTAA...LTIDTETSVETSLVDPSRDVT
CsPDCE1a	TTFPELITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
PhPDCE1a	TNNEPLITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
VvPDCE1a	TTFPELITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
GhPDCE1a	TNNEPLITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
BrPDCE1a	SSTQELITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
AtPDCE1a	SSSQELITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
GmPDCE1a	TSSQELITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
PsPDCE1a	TSPSELITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
OsPDCE1a	TIARELATFPRDSAMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITKKICITAYRDHCIFL
ZmPDCE1a	ITFPELITFPRDVALMRR.EIADSLYKAKLIRGFCHLYDGQEAVALGMPAAITRSISITAYRDHCIFL
TPP – Binding site	
CsPDCE1a	GRGGTLEAFELMGRKAGCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDEEHTFTM
PhPDCE1a	SRGGTLEFCESELMGRKIDCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDEEHTFTM
VvPDCE1a	GRGGTLEVECESELMGRKAGCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDEETVTFM
GhPDCE1a	SRGGTLEVEFELMGRKAGCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDEETVTFM
BrPDCE1a	GRGCSLYEVEFELMGRKIDCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDEEAVTFM
AtPDCE1a	GRGCSLYEVEFELMGRKAGCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDEEAVTFM
GmPDCE1a	GRGGTLEVEFELMGRKIDCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDESVTFM
PsPDCE1a	GRGGTLEVRVAFELMGRKIDCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDESVTFM
OsPDCE1a	ARGGDTAALFELMGRKAGCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDEAAVTFM
ZmPDCE1a	ARGGDTVSAFELMGRKIDCSRGKGGSMHFYKRDSEFYGGHGIVGAQVPLGGLAFACQVSRDEETVTFM
PDH β – Binding site	
CsPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
PhPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
VvPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
GhPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
BrPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
AtPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
GmPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
PsPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
OsPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
ZmPDCE1a	YGDGAANQGQLFEALNIPALNDLEALVLCENNHYGMGTAEWRAAKSFYWKRGDYPVGLKVDGMVIAVK
* *	
CsPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
PhPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
VvPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
GhPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
BrPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
AtPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
GmPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
PsPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
OsPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
ZmPDCE1a	QAQFAKPHAKNGHIILEMDTYRYHGHSMSPDGSTYRTRDEISGRQERDPIERIKKILVSDIATEKE
* *	
CsPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
PhPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
VvPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
GhPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
BrPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
AtPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
GmPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
PsPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
OsPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL
ZmPDCE1a	LKMEKEIRKEVDIAIAKREIEMEDFSLEFNVYVKGFGTSGAGADRKEVRATL

180x305mm (300 x 300 DPI)

47x29mm (300 x 300 DPI)

33x10mm (300 x 300 DPI)

79x106mm (300 x 300 DPI)

207x72mm (300 x 300 DPI)

Review Only

33x21mm (300 x 300 DPI)