Available online at www.sciencedirect.com
ScienceDirect
Plant Physiology and Biochemistry xx (2007) 1–10
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Research article

Sucrose regulated expression of a Ca^{2+}-dependent protein kinase (TaCDPK1) gene in excised leaves of wheat

Giselle Martínez-Noël [a,*], Vinay J. Nagaraj [b], Gonzalo Caló [a], Andres Wiemken [c], Horacio G. Pontis [a]

[a] Centro de Investigaciones Biológicas, Fundación para Investigaciones Biológicas Aplicadas (FIBA), Vieytes 3103, 7600 Mar del Plata, Argentina
[b] The Biodesign Institute at Arizona State University, Tempe, AZ 85287, USA
[c] Botanisches Institut der Universität Basel, Hebelstrasse 1, CH 4056 Basel, Switzerland

Received 19 January 2007; accepted 5 March 2007

Abstract

Sucrose (Suc) can influence the expression of a large number of genes and thereby regulates many metabolic and developmental processes. However, the Suc sensing and the components of the ensuing signaling transduction pathway leading to the regulation of gene expression are not fully understood. We have shown that protein kinases and phosphatases are involved in the Suc induced expression of fructosyltransferase (FT) genes and fructan accumulation by an hexokinase independent pathway in wheat (*Triticum aestivum*). In the present study, using an RT-PCR based strategy, we have cloned a calcium-dependent protein kinase (TaCDPK1) cDNA that is upregulated during Suc treatment of excised wheat leaves. The deduced amino-acid sequence of CDPK1 has high sequence similarity (>70%) to known CDPKs from both monocots and dicots. Based on sequence homology, TaCDPK1 sequence shows a variable domain preceding a catalytic domain, an autoinhibitory function domain, and a C-terminal calmodulin-domain containing 4 EF-hand calcium-binding motifs, along with a N-myristoylation motif in the N-terminal variable domain. The recombinant *Escherichia coli* expressed TaCDPK1 was able to phosphorylate histone III-S in a calcium dependent manner in in vitro assays. The TaCDPK1 gene expression, as determined by quantitative RT-PCR, is induced by Suc and this effect is repressed by the inhibitors of the putative components of the Suc signal transduction pathway (calcium, Ser/Thr protein kinases and protein phosphatases). We propose that TaCDPK1 is involved in the Suc induced signaling pathway in wheat leaves.
© 2007 Published by Elsevier Masson SAS.

Keywords: Calcium-dependent protein kinase; Fructan; Sugar signaling; *Triticum aestivum*

1. Introduction

Several physiological processes, such as cellular metabolism, cell division, growth and differentiation, are regulated by reversible phosphorylation of specific cellular proteins in response to an array of diverse external and internal stimuli. These are signals that plant cells have to sense to survive and adapt; they include light, temperature, gravity, mineral nutrients, environmental stresses, fungal elicitors, cell damage, developmental processes, phytohormones, and organic metabolites like peptides and sugars [1,2]. The relay of these signals commonly involves protein kinases which catalyze the phosphorylation of enzymatic and non-enzymatic proteins and thereby alter activity or interaction properties of the target proteins [3].

Protein kinases regulated by cytosolic free calcium (Ca^{2+}) are key components for signal transduction pathways in all eukaryotes [4,5]. The predominant Ca^{2+}-stimulated protein kinase

Abbreviations: CDPK, calcium-dependent protein kinase; Suc, sucrose; BAPTA, bis-(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $LaCl_3$, lanthanum chloride; RR, rutenium red; 6-SFT, 6-sucrose:fructan fructosyltransferase; 1-SST, 1-sucrose:sucrose fructosyltransferase; ST, staurosporine; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; OA, okadaic acid.

* Corresponding author. Tel.: +54 223 475 1453; fax: +54 223 475 7120.
E-mail address: gnoel@fiba.org.ar (G. Martínez-Noël).

0981-9428/$ - see front matter © 2007 Published by Elsevier Masson SAS.
doi:10.1016/j.plaphy.2007.03.004

UNCORRECTED PROOF

activity in plant extracts is attributed to Ca^{2+}-dependent protein kinases (CDPKs), a group of enzymes identified only in plants and some protists [6,7]. Plant CDPK family is represented by many genes, with 12 subfamilies comprising 34 isoforms in *Arabidopsis* and 27 in rice [8,9]. CDPKs may function as a potential sensor that decodes and translates Ca^{2+} concentration increase into enhanced protein kinase activity and subsequently trigger downstream signaling events [9,10]. CDPKs have a highly conserved structure which contains three domains with well-characterized functions: Ser/Thr kinase catalytic, autoregulatory and Ca^{2+}-binding domains [6,7]. Therefore, CDPKs do not depend on the interaction with exogenous calmodulin but could be activated directly by Ca^{2+} binding. The autoinhibitor domain functions as a pseudo-substrate that inhibits phosphorylation in the absence of Ca^{2+} and keeps the CDPK in a low activity state [11]. Isoform-specific differences among CDPKs are mainly restricted to the N-terminal variable domain, in which many of them also include a fatty acylation site. Myristoylation and palmitoylation at this site can act as membrane anchor and determine localization of CDPKs [11,12]. The individual isoforms have different functions and participate in multiple signaling pathways [10].

Sugars are not only important energy sources and structural components, they are also central regulatory molecules controlling metabolism, the cell cycle, development, and gene expression [13,14]. While the expression of a variety of plant genes is known to be influenced by the level of sugars such as Suc, glucose or fructose, neither the exact sensing mechanisms nor the sugar-generated signaling events leading to gene expression changes are fully understood. We have previously reported that the sugar-inducible expression of genes for the fructan synthesis [1 sucrose:sucrose fructosyltransferase (1-SST, EC 2.4.1.99) and 6-sucrose:fructan fructosyltransferase (6-SFT, EC 2.4.1.10)] in wheat leaves decreased in the presence of inhibitors of Ser/Thr protein kinase, calmodulin and Ca^{2+}-channels [15]. Our observations pointed toward an important role for CDPKs in plant sugar signaling pathways. With the aim to identify the precise components of sucrose signaling pathway, we focused on the study of kinases that are upregulated by Suc treatment in wheat leaves. In the present study, we report the cloning and heterologous expression of a CDPK cDNA from wheat (designated TaCDPK1) and the regulation of TaCDPK1 gene expression by Suc. We propose that TaCDPK1 is involved in the Suc induced signaling pathway in wheat leaves.

2. Materials and methods

2.1. Plant material and leaf treatments

Wheat (*Triticum aestivum* L.) seeds of winter type Pincén cultivar were germinated and grown for 8 days on vermiculite, in a controlled environment room at 27 °C, 250 μmol m^{-2} s^{-1} photosynthetic photon flux density, a day/night regime of 16/8 h and watered daily with one half strength Hoagland solution. Primary leaf blades were excised and placed in tubes with the proximal ends immersed in 500 μl of 200 mM Suc solutions in the presence or absence of inhibitors for 24 h in darkness. For the experiments with inhibitors the leaves were pretreated with the inhibitor alone for 2 h or with water (control). We used the following chemicals: 1,2-bis(2-aminophenoxyethano)-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), $LaCl_3$ (La^{3+}), ruthenium red (RR), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), staurosporine (ST), and okadaic acid (OA) at 5 mM, 10 mM, 50 μM, 200 μM, 2 μM and 1 μM respectively. After harvesting, the leaf blades were frozen in liquid nitrogen and stored at −80 °C.

2.2. Cloning of TaCDPK1 cDNA

Using the highly conserved amino acid residues DLKPENF and ADIWTAG found in the catalytic domain of the CDPK sequences (VIb and IX subdomain, respectively), the degenerate primers, dCDPKFor (GAY YTI AAR CCI GAR AAY TT) and dCDPKRev (CCI GCI GTC CAD ATR TCI GC), were designed. Total RNA was isolated from wheat leaves treated with Suc 200 mM for 24 h in the dark with the Trizol reagent (Invitrogen). After treatment with DNaseI (MBI Fermentas), cDNA was synthesized from 1 μg of total RNA using a Reverse Transcription Kit (Promega). The cDNA was used as a template for PCR with the dCDPKFor and dCDPKRev primers to amplify a 192-bp fragment, which was cloned into pGEM-Teasy (Promega). Several positive clones were sequenced and all contained the same sequence, with high similarity to plant CDPKs. This nucleotide sequence was used to search for homologous wheat ESTs in public DNA databases. The matching EST sequences (BQ744288, BJ270434, CD896363, CD896110 and CD933325) were collected and assembled in silico to obtain a 1828-pb cDNA sequence that corresponds to the putative full-length CDPK, which was used to design the flCDPKFor (TCG TGC CGC TCA ATC TCC CCT CGT CAA A) and flCDPKRev (CTT AAC ATA ATC ATT AAA AAA CTG TTA CAC CAC GCC TGT TTC) primers. These primers were used for PCR amplification of the CDPK sequence from cDNA using the Expand Long Template PCR kit (Roche Diagnostics Corporation) according the manufacturer's protocol with 63 °C as the annealing temperature. The amplified fragment was cloned into pGEMT and sequenced. The location of the various primers on the TaCDPK1 sequence is indicated in Fig. 1.

2.3. Analysis of gene expression

RNA was isolated from leaf tissue using the RNeasy Plant Mini kit (Qiagen). The RNA was treated with DNase I (MBI Fermentas) to remove contaminating DNA, subjected to phenol/chloroform extraction and quantified spectrophotometrically. One μg of RNA was used for synthesis of cDNA using the Reverse Transcription System (Promega). The cDNA was used as a template for RT-PCR and Real-time PCR reactions (see below). For the analysis of the TaCDPK1 gene expression, rtCDPKFor (5′ AAT CGG CGG CGC ATG TTC TA 3′) and rtCDPKRev (5′ GCA GCA ATG GAA CCG ATC ATG T 3′) primers that can amplify a 159-bp sequence

Please cite this article in press as: G. Martínez-Noël et al., Plant Physiol. Biochem. (2007), doi:10.1016/j.plaphy.2007.03.004

```
tcgtgccgctcaatctcccctcgtcaaacccattcacacacaatgggcaaccgcacctcg 60
                                           M  G  N  R  T  S*
cgccaccgccgcgccgccgcggaccagccggccacggcccctccgccgacggcccagccc 120
 R  H  R  R  A  A  A  D  Q  P  A  T  A  P  P  P  T  A  Q  P
aaaccccagccgccgccgaagccccagacggcgccggccccggccccgacgccggaagcg 180
 K  P  Q  P  P  P  K  P  Q  T  A  P  A  P  A  P  T  P  E  A
ggacaggtggccatgggccgcgttctgggacgcccgatggaggacgtgcgcgcgacctac 240
 G  Q  V  A  M  G  R  V  L  G  R  P  M  E  D  V  R  A  T  Y
accttcggccgcgagctcggccgggggcagttcggggtcacctacctcgtcacgcacaag 300
 T  F  G  R  E  L  G  R  G  Q  F  G  V  T  Y  L  V  T  H  K
gctacgggccagcgcttcgcctgcaagtccatcgccacgcggaagctcgtccaccgcgat 360
 A  T  G  Q  R  F  A  C  K  S  I  A  T* R  K  L  V  H  R  D
gacatcgaggatgtgcagcgggaggtgcagatcatgcaccacctcacgggtcaccgcaac 420
 D  I  E  D  V  Q  R  E  V  Q  I  M  H  H  L  T  G  H  R  N
atcgtcgagctccgcggcgcctacgaggaccgccactcggtcaacctcatcatggagcta 480
 I  V  E  L  R  G  A  Y  E  D  R  H  S* V  N  L  I  M  E  L
tgtgagggcggggagctattcgaccgcatcatcgcgcgggggcactactccgagcgagcc 540
 C  E  G  G  E  L  F  D  R  I  I  A  R  G  H  Y  S* E  R  A
gccgccctgctctgccgcgagatggtctccgtcgtgcacagctgccactccatggggtt 600
 A  A  L  L  C  R  E  M  V  S  V  V  H  S  C  H  S  M  G  V
ttccaccgggatctcaagcccgagaacttttgtttctcaacaacaaggaggactcgccg 660
 F  H  R  D  L  K  P  E  N  F  L  F  L  N  N  K  E  D  S* P
ctcaaggccactgacttcggtctctccgtcttcttcaaacacggggagcagtttaaggat 720
 L  K  A  T  D  F  G  L  S  V  F  F  K  H  G  E  Q  F  K  D
ctcgttggaagtgcgtattatgttgctcctgaggtactgaagcggcactatggagcagaa 780
 L  V  G  S  A  Y  Y  V  A  P  E  V  L  K  R  H  Y  G  A  E
gctgacatatggagtgcggggattattctgtacatccttctgtctggtgttcctcctttt 840
 A  D  I  W  S  A  G  I  I  L  Y  I  L  L  S  G  V  P  P  F
tgggcagacaatgaggatggcatatttgaggctgtcttgcttggtcacatagatttctca 900
 W  A  D  N  E  D  G  I  F  E  A  V  L  L  G  H  I  D  F  S
tctgatccctggccttcaatatctaatggtgcaaaagatttggtgaagaagatgttgcgg 960
 S  D  P  W  P  S* I  S  N  G  A  K  D  L  V  K  K  M  L  R
caagaccccaaagagcgcttgactgctgcagaaattttgaatcacccatggattagggaa 1020
 Q  D  P  K  E  R  L  T* A  A  E  I  L  N  H  P  W  I  R  E
gatggagaggctccagataagccacttgacattactgttatcagtagaatgaagcagttc 1080
 D  G  E  A  P  D  K  P  L  D  I  T  V  I  S  R  M  K  Q  F
cgggcgatgaacaagcttaagaaagttgcattgaagatcgttgcagagaacttgtctgag 1140
 R  A  M  N  K  L  K  K  V  A  L  K  I  V  A  E  N  L  S* E
gaagagataacaggcttgaaagaaatgttcagatccctggacactgataacagtgggaca 1200
 E  E  I  T  G  L  K  E  M  F  R  S  L
attactcttgaagagctaagatctggtttaccaaaacttggcaccaaaatttctgaatca 1260
                   R  S  G  L  P  K  L  G  T  K  I  S* E  S
gaaattacacagttaatggaggcggctgatgttgatggaaatgggaccattgattattct 1320
 E  I  T  Q  L  M  E  A  A  D  V  D  G  N  G  T  I  D  Y
gagttcgtatcagcgacaatgcacatgaatagattggagaaggaagaccacatacttaaa 1380
       V  S  A  T  M  H  M  N  R  L  E  K  E  D  H  I  L  K
gcatttgaatatttgataaggaccacagcggatacataacagtagatgagctggaagaa 1440
 A  F  E  Y* F                                            E  E
gctctgaagaagtatgacatgggggatgataaaacaattaaagatattatcgctgaagtt 1500
 A  L  K  K  Y  D  M  G  D  D  K  T* I  K  D  I  I  A  E
gatacagatcatgatggaagaatcaactaccaggagttcgttgccatgatgagaaacaac 1560
                                        V  A  M  M  R  N  N
agcccggagattgttccaaatcggcggcgcatgttctaattatcttctaccgtaacatct 1620
 S* P  E  I  V  P  N  R  R  R  M  F  -
tcctggccccattatcgtccaagaataattttgagggatgtgccgctttgatgttattgt 1680

ccctcatctttagatttggcgtgtatttccttgatcacatgatcggttccattgctgctc 1740

cgagggtattatgatcatatgcgtactcctcctgatcatttatatggaaacaggcgtggt 1800

gtaacagtttttaatgattatgttaag                                  1828
```

Fig. 1. cDNA sequence of cloned TaCDPK1 with the deduced amino-acid sequence. The underlined nucleotides at the 5′ and 3′ ends correspond to the sequence of the primers used for cloning of the 1828 bp TaCDPK1 cDNA sequence (flCDPKFor and flCDPKRev). The nucleotides in bold correspond to the primers used for subcloning into the *E. coli* expression vector (ExpCDPKFor and ExpCDPKRev). The highlighted grey nucleotides in italics correspond to the primers used for TaCDPK1 RT-PCR and Real-time PCR studies (rtCDPKFor and rtCDPKRev). The individually underlined amino acids are the conserved region used for making degenerate primers for cloning the partial CDPK sequence (dCDPKFor and dCDPKRev). The highly conserved amino acid domains are denoted as follows; N terminal variable region, underlined; myristoylation motif, grey shading; kinase domain, bold letters; auto inhibitory domain, grey letters; predicted phosphorylation sites, with asterisk and EF hands, black shading.

```
                     *        20         *        40         *        60         *        80
P53683 :   SCCSRATSPDSGRGGANGYGYSHQTKPAQTTPSYNHPQPPPPAEVRYTPS--------------AMNPPVVPP------ :  61
P49101 : ---MVMAILTRQSRR---------------KHLRVYNPPQQ--AAEVRYTPS--------------ATNSSAVPP------ :  41
O24431 :  VTDMLGLVVCTTKK--------THEPLVNQSRAPANQPYHLHEKHAASTAQT------------VPQNMPWKPPG----- :  56
O81390 :   GCFSKKYRQEGANGG-----YRATRRNANQEYQKPPQHQPERPYQPQPQP------------QPQPQPRPQPQAHTVT :  63
Q8RW36 :   GCFSKKYTQQDANG-------HRAGRR-VNQAYQKPPQPQPERPYQPQPQQERPYQPPPQPAYQPPPQPKPQPQPHPVP :  73
Q9ZPM0 :   ICASKNKATQPEDNG-------YTPANGVGSNNQSKPPAAQQNYHQQPPQQP------------VAPPQPASHPP----- :  57
Q6KCK6 :   NRTSRHRRA-------------AADQPATAPPP---TAQPKPQPPPKPQTA-------------PAPAPTPEAG----- :  47
Q42479 :   HRHSKSKSS-------------DPPPSSSSSSSGNVVHHVKPAGERRGSSG-------------SGTVGSSGSGT---- :  51
Q9ARI5 :   HPLDHSDAH-------------HRPPSNG-------VIKVLPPDSDPSLPP-------------PLPKQLPTSS----- :  43
Q3YAS9 :   NCCSRGQPNESND---------HTESISTAKNGDGKVMQGGSVTPPKTPPP-------------ASPDPSSKPS----- :  54

                *       100        *       120        *       140        *       160
P53683 : ----VVAPPKPTP----DT     YD    SV  L             C EIA   KQ       SK     S A K         : 134
P49101 : ----VAVPPKPTA----DT     QY    SV  F             C EIA   RQ       SK     S A R         : 114
O24431 : ----PALSPKPVVGVRQDT     QF    QFH L             C ENS   LQ       SK    AS S K         : 133
O81390 : ---QPXQTQDQMQGPHLNN      F    KQ  L            HC ENS   NP       LK     R N R         : 142
Q8RW36 : VTVQSGQPQDQMQGPHMNN      F E  KL  L            YC ENS   NP       LK     S N R         : 154
Q9ZPM0 : ---VRMPSPKPAPKVEPNT      F    VY  L             C DKK   QQ       SK     T A KD        : 135
Q6KCK6 : -------------QVAMGR      M    AT  F             V HKA   QR       AT     H D I    Q    : 115
Q42479 : -----GGSRSTTSTQQNGR      M E  RT EF             V HKE KQQV       PT     H D I         : 127
Q9ARI5 : ------------APPALGR  A  FS    SF  FRG           V HKE KQD        AT     DQD V         : 112
Q3YAS9 : ------------KTSPIGP      M    KT  I            H C HKQ  EQ       AK     N E I         : 123

               *       180        *       200        *       220        *       240
P53683 :    Q     QQ     F       SN H                    H    A  T C A  N  NI  F              : 215
P49101 :    Q     QP     F       SN H                    H    A  T C A  N  NI  F              : 195
O24431 :    Q     QP     F       SS H                    H    A  S C Q  N  HI  F              : 214
O81390 :    Q     QP     F       HS H                    Y    D  E I Q AN  NI  F              : 223
Q8RW36 :    Q     QP     F       QS H                    Y    D  E I Q  N  NI  F              : 235
Q9ZPM0 :    Q     QP     F       TS N                    H    A  T L Q  N  HV  F              : 216
Q6KCK6 :    H     HR     L       HS N      E             H    A  L C E  S  HS  S     F        : 196
Q42479 :    H     HR    DL       HS N      E        S    L    A  D C Q  M  HS  S              : 208
Q9ARI5 :    H     HPH    L EV    HY N                    H   CT  S C Q  T  HN  S              : 193
Q3YAS9 :    H     QQ     L       HS N                    H    A  T L T  Q GHT  S              : 204

              *       260        *       280        *       300        *       320
P53683 :  LA KE   AM            IEE K                   N  K I                       T K         : 296
P49101 :  LA ME   AM            IEE K                   S  K I                       I K         : 276
O24431 :  LS RD   AL            IEE K                   RC K I                       T K         : 295
O81390 :  LT KD   AK            IEE K                   S  K A                       T K         : 304
Q8RW36 :  LT KD   AM            IEE K                   S  K A                       T K     T   : 316
Q9ZPM0 :  LS KD   SL            IEE K                   R  K I                       T K         : 297
Q6KCK6 :  FLNNK   SP            FKH EQ                  H  A A                     DN D    E    : 277
Q42479 :  FL KD   SP            FKP DK                  N  P A                     G N T         : 289
Q9ARI5 :  FL AD   SP            FKP D                   R  P AA                      NDQ         : 274
Q3YAS9 :  LLNKD   SP            YKQ D                   R  P V     V                 T H         : 285

             *       340        *       360        *       380        *       400
P53683 :   Q E    E Q    S   ES          TQ    K    S     Q      DG-         S                    : 376
P49101 :   HEE    E Q    S   ES          TR    K    S     Q Q    EGG         S                    : 357
O24431 :   E H    E Q    N   DS          IQ    K    S     E      DG-N        S                    : 375
O81390 :   K E    Q D    S   NS          TQE   K    S     E      MG-         S                    : 384
Q8RW36 :   K E    Q D    S   NS          TQE   K    S     E      LG-         S                    : 396
Q9ZPM0 :   Q H    E K    S   NG          TQ    K    A     E      DG-         S    L       V       : 377
Q6KCK6 :   L H    S D    S   NG          RQ    E    A     N      EDG   P     IT                   : 358
Q42479 :   Q Q    SAD    A   DG          KY    D    A     N      EDG         N                    : 370
Q9ARI5 :   R H    S D    S   SG          HS    E    AI A  N      EDG         I                    : 355
Q3YAS9 :   R H    SCD    A   HG          TI    Q    AI    N      EDGD P T    N              NF    : 366

            *       420        *       440        *       460        *       480
P53683 :    S   N     K   Q   TN             Y    A   A         A                  V         RH    : 457
P49101 :    S   N     K   Q   MN             Y    A   A         A                  V         RH    : 438
O24431 :    E   SA    Q   A   TN     K       Y    S   H         A                  I         RH    : 456
O81390 :    E   S     K   A   AN             Y    S   A         T                  I         RH    : 465
Q8RW36 :    E   S     K   A   HN             Y    S   A         T                  I         RH    : 477
Q9ZPM0 :    E   PD    Q   Q   AN     G       Y    E   A         T    A      Q  S   Y         RY    : 458
Q6KCK6 :    E   S     T   E   RS             L    S   P         S    T             S         MN    : 439
Q42479 :    E   S     I   E   KS        N I  L    T   P         A                  L         MN    : 451
Q9ARI5 :    E   S     V   E   KS             F    A   P         S                  I     A   MN    : 436
Q3YAS9 :    GC  S     M   Q   KS        A    L    T   A Q     DY   Q       A       E         MN  D : 447

           *       500        *       520        *       540        *       560
P53683 :  DE           N      RD    S   IEHE    TS    D   S    -------   N       E   C     G GM QPMR : 531
P49101 :  DE           N      RD    S   IEHE    TS    E   S    -------   N       E   C     G -M QPMR : 511
O24431 :  DDQ          N      RD    S    E G    DA    E   S    TIISEVD   H       E   S     S NQ QGKL : 537
O81390 :  DE           H      RD    S    E G    EA    E   A    -------   N       E   C     S -T PQPK : 538
Q8RW36 :  DE       H   H      RD    N    E G    EA    E   A    -------   N       E   C     S TT PQQK : 551
Q9ZPM0 :  DE           N      TD    T    E G A  ENC  AE   L    -------   N       D   S     S TQNPVTK : 532
Q6KCK6 :  ED  L        H      VD    E    K D    DK    D   A    -------   H       Q   V    NNSP IVPN : 513
Q42479 :  ED    T     N N     ME    L    K N    DK    E   A    -------   R       E   V     K NP LVPN : 525
Q9ARI5 :  ED           K      ME    T    K N    ET    E   A    -------   N       D  VV     K NQ LTTT : 510
Q3YAS9 :  EE    T      N      VE    Q    E G T  GKD   D   A    -------   N       D   V     K TPDTAAN : 521

          *
P53683 : LK--------- : 533
P49101 : LK--------- : 513
O24431 : F---------- : 538
O81390 : LF--------- : 540
Q8RW36 : LF--------- : 553
Q9ZPM0 : LI--------- : 534
Q6KCK6 : RRRMF------ : 518
Q42479 : RRRM------- : 529
Q9ARI5 : SRQK------- : 514
Q3YAS9 : LKKRRESFDAK : 532
```

corresponding to the 3′ region of TaCDPK1 were designed. Ubiquitin (Ubi) was used as a control for gene expression analysis. Ubi transcript levels were analyzed using the primers rtUbiFor 5′-CCT TCA CTT GGT GCT CCG TCT-3′ and rtUbiRev 5′-AAC GAC CAG GAC GAC AGA CAC A-3′ (PCR product size 152 bp).

For conventional RT-PCR, cDNA was directly used as a template for PCR with Taq polymerase (Promega) for 25 cycles. PCR products were run on an ethidium bromide containing 1% agarose gels. Real-time PCR was performed with a Gene Amp 5700 Sequence Detection System (Applied Biosystems). The thermal profile was, 1 cycle 2 min at 50 °C, 1 cycle 10 min at 95 °C, 40 cycles 15 s at 95 °C, 58 °C 15 s and 1 min at 60 °C. A 25 μl reaction volume consisted of 12.5 μl SYBR Green PCR master mix (Applied Biosystems), 0.3 μM gene specific forward primer, 0.3 μM gene specific reverse primer and 1 μl of cDNA preparation (diluted 1:5). Copy numbers were calculated from amplification plots of known standards for all the genes. Ubi transcript levels in the different samples were used to normalize the amounts of CDPK1.

2.4. Production of recombinant TaCDPK1 in E. coli

The region corresponding to the open reading frame (ORF) of TaCDPK1 cDNA was PCR amplified from the full length cDNA clone using ExpCDPKFor (CCG **CTC GAG** ACA ATG GGC AAC CGC ACC T) and ExpCDPKRev (G**GA ATT C**CAA CAT CAA AGC GGC ACA TCC CTC) primers containing the restriction sites (in bold) for *Xho*I and *Eco*RI respectively (Fig. 1). The amplification products were digested with *Xho*I and *Eco*RI, and ligated to pRSETA (Invitrogen) which was previously linearized using the same restriction enzymes. The resulting clone (pRSETA-TaCDPK1) contains the TaCDPK1 ORF under the control of the bacteriophage T7 promoter whose expression is induced by the production of T7 RNA polymerase in *E. coli*. The construct was sequenced to check for cloning errors. The pRSETA-TaCDPK1 expression construct was introduced into BL21(DE3):pLysS strain of *E. coli* (Stratagene) where the expression of the T7 RNA polymerase can be induced by isopropyl-1-thio-β-galactopyranoside (IPTG).

Transformed BL21(DE3):pLysS was cultured in the presence (induced) or absence (non-induced) of 1 mM IPTG for 16 h at 25 °C. The cells were harvested by centrifugation (5000 × *g* for 15 min), washed twice in 100 mM Hepes (pH 7.5), 600 mM NaCl, 15 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and re-suspended in the same buffer. The suspensions were frozen in liquid nitrogen and thawed three times to lyse the cells. Then, they were treated with 10 μg ml^{-1} DNase/RNase on ice for 30 min and were passed five times through syringes. The cell lysate was centrifuged at 12,000 × *g* for 20 min at 4 °C to pellet the cell debris. Protein concentration in the supernatant was determined [16] and the extract was used for further analysis.

2.5. Immunoblotting

The soluble proteins from transformed *E. coli* were resolved on a 10% polyacrylamide gel containing SDS and stained with Coomassie blue or electroblotted onto a nitrocellulose membrane (HyBond C, Amersham). The membranes were then probed with polyclonal antibodies raised against the calmodulin-like domain of soybean (*Glycine max*) CDPK (dilution 1/1000). The kinase was visualized using the 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate system according to the manufacturer's directions.

2.6. Protein kinase activity assay

Protein kinase activity of the recombinant TaCDPK1 was assayed in vitro by analyzing the transfer of radioactivity from [γ^{32}P]ATP to histone type III-S (Sigma) in cell-free extract of transformed *E. coli*. Protein kinase assay was performed as described by Saijo et al. [17]. The reaction mixture was separated on a 10% SDS-PAGE and subjected to autoradiography.

3. Results

3.1. Identification and cloning of a CDPK cDNA from wheat leaves

In previous studies on the Suc signaling pathway responsible for the activation of FT genes in wheat leaves, we have shown that CDPK activity is essential for the signal transduction processes [15]. Using degenerate RT-PCR primers based on the highly conserved protein kinase catalytic domain of CDPKs, we observed that Suc feeding of excised wheat leaves resulted in an increase in CDPK gene expression (data not shown). This DNA fragment amplified by RT-PCR (192 bp) was cloned and sequencing of several independent clones resulted in only one identical sequence with a high similarity to known plant CDPKs. Next, wheat EST sequences, available in public databases, that matched the cloned CDPK fragment were collected and assembled in silico to obtain a 1828-bp sequence. This sequence was used to design specific primers to amplify the in silico predicted sequence, using previously prepared cDNA as template for the PCR. The PCR product was cloned and its sequence was found to contain the 192 bp sequenced earlier. The newly cloned cDNA was designated as TaCDPK1 and the sequence has been deposited to the EMBL database (accession number AJ621356).

Fig. 2. Comparison of amino-acid sequence of the newly cloned TaCDPK1 from *Triticum aestivum* (Q6KCK6) with well characterized CDPKs from other plants; *Arabidopsis thaliana* (Q42479), *Cucumis sativus* (Q9ARI5), *Petunia inflate* (Q3YAS9), *Nicotiana tabacum* (O81390), *Oryza sativa* (P53683), *Lycopersicon esculentum* (Q8RW36), *Zea mays* (P49101), *Mesembryanthemum crystallinum* (Q9ZPM0), *Glycine max* (O24431). Identical amino acids are shade in black and highly conserved residues are shaded in gray.

UNCORRECTED PROOF

Please cite this article in press as: G. Martínez-Noël et al., Plant Physiol. Biochem. (2007), doi:10.1016/j.plaphy.2007.03.004

3.2. Sequence analysis of TaCDPK1 from wheat leaves

Based on sequence comparisons, the 1828-bp fragment of TaCDPK1 appears to contain the entire CDPK coding region. The TaCDPK1 cDNA includes a 5′ 42 bp untranslated region, a 1557 bp open reading frame (ORF) and a 229 bp 3′ untranslated region (Fig. 1). The peptide sequence derived from the cDNA consists of 518 amino acids with a predicted Mr of 58.4 kDa and an isoelectric point of 5.8.

The length and amino acid composition of the N-terminal region of plant CDPKs is less conserved. However, the N-terminal region of CDPKs tends to be rich in proline and glutamine residues. The deduced amino-acid sequence of TaCDPK1 contains a N-terminal region that is 53 residues long and contains a proline-glutamine rich sequence, with the first eight amino acids containing a putative myristoylation site (MGXXXSXX) (Fig. 1). The TaCDPK1 protein has the domains typically found in all known CDPKs; a kinase domain consistent with the structure of Ser/Thr protein kinases (amino acids 66–324), connected to a calmodulin-like domain with four EF-hand calcium-binding domains (amino acids 380–392, 416–428, 452–464 and 487–499) by a short 'junction domain' (amino acids 328–360) (Fig. 1). An alignment of the TaCDPK1 amino-acid sequence with other highly similar and very well characterized CDPKs reveals that all the above mentioned domains are highly conserved (Fig. 2).

Rice and Arabidopsis genomes contain numerous isoforms of putative CDPKs. Based on differences in their regulatory domains, the different CDPKs isoforms have been classified into subgroups [6,18]. Phylogenetic analysis indicates that TaCDPK1 protein belongs to the subgroup IIa of the CDPK family (Fig. 3). Seven other CDPK-like sequences have been

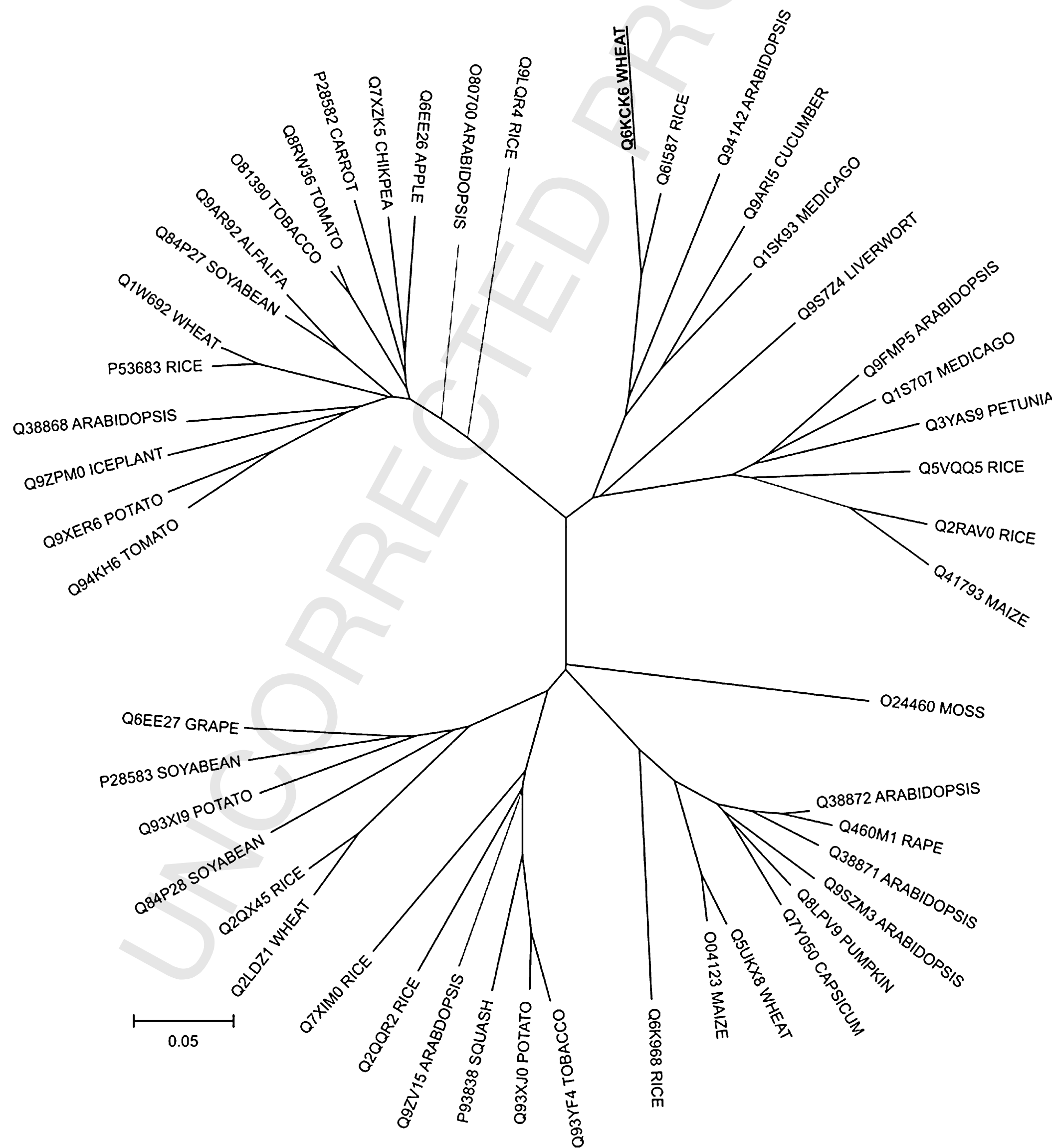

Fig. 3. Unrooted phylogenetic tree depicting the relationship of TaCDPK1 protein sequence (QKCK6 wheat, underlined) with closely related CDPKs from different plant species. The sequences are named after the EMBL database protein accession number followed by the common name of the plant. The top and lower branches of the tree represents sequences from CDPK group II and I respectively [17] with TaCDPK1 clustering with group IIb members. The sequences were aligned and phylogenetic tree constructed using Mega 3.1 sequence analysis software [38].

UNCORRECTED PROOF

Please cite this article in press as: G. Martínez-Noël et al., Plant Physiol. Biochem. (2007), doi:10.1016/j.plaphy.2007.03.004

reported from wheat (Q2LDZ3, Q1W693, Q2LDZ2, Q1W692, Q5UKX8, Q2LDZ1 and Q2LDZ0). While gene duplication is responsible for the multiple isoforms of CDPKs [6,8], it is not clear which wheat sequence may be more closely related to the common ancestor. It is also interesting to note that clustering of CDPK sequences is completely species independent and highly influenced by the sequence of the functional domains. This has led to suggest that CDPK isoforms that make up a subgroup of the phylogenetic tree may involved in similar cellular signaling pathways [10].

3.3. *Effect of sucrose on TaCDPK1 transcript levels in wheat leaves*

TaCDPK1 gene expression was investigated by RT-PCR and Real-time PCR to quantify differences in transcript levels among samples. Specific primers were designed based on the newly cloned TaCDPK1 sequence.

TaCDPK1 appears to be constitutively expressed in leaves as well as roots (Fig. 4A). On the other hand, leaves incubated in 200 mM Suc for 24 h showed increased levels of TaCDPK1 mRNA (Fig. 4B). TaCDPK1 expression was also analyzed using inhibitors of the putative components of the Suc signaling pathway (Ca^{2+}, Ser/Thr protein kinases and protein phosphatases 2A/1). We used the specific Ca^{2+} chelator bis-(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), the Ca^{2+} channels inhibitors $LaCl_3$ (La^{3+}) and ruthenium red (RR), the inhibitor of Ser/Thr protein kinases staurosporine (ST), the calmodulin antagonist W7 and the inhibitor of protein phosphatases 2A/1 okadaic acid (OA). We previously demonstrated that these inhibitors reduced or blocked the expression of FT genes by Suc [15]. Real-time PCR results showed that all of the inhibitors tested were able not only to block the Suc induced upregulation of the TaCDPK1 but also interestingly, to decrease the basal level of transcripts as compared to control leaves (Fig. 4B). It is noteworthy that TaCDPK1 transcripts were very low in samples treated with OA.

3.4. *Heterologous expression of TaCDPK1 cDNA in E. coli*

To analyze the activity of the TaCDPK1 gene product, we subcloned the ORF region of the cDNA into the expression vector pRSET-A. The resulting expression construct was sequenced and introduced into the *E. coli* strain BL21(DE3)-pLysS. Soluble protein extracts from transformed *E. coli*, induced for transgene expression, were submitted to SDS-PAGE and western blot analysis. Antibodies against the calmodulin-like domain of a soybean CDPK revealed a polypeptide of molecular mass comparable to that of TaCDPK1 gene product (Fig. 5A).

As shown in Fig. 5B, the recombinant TaCDPK1 efficiently phosphorylates the exogenous substrate histone III-S in the presence of Ca^{2+} and the enzyme activity is almost completely inhibited by EGTA.

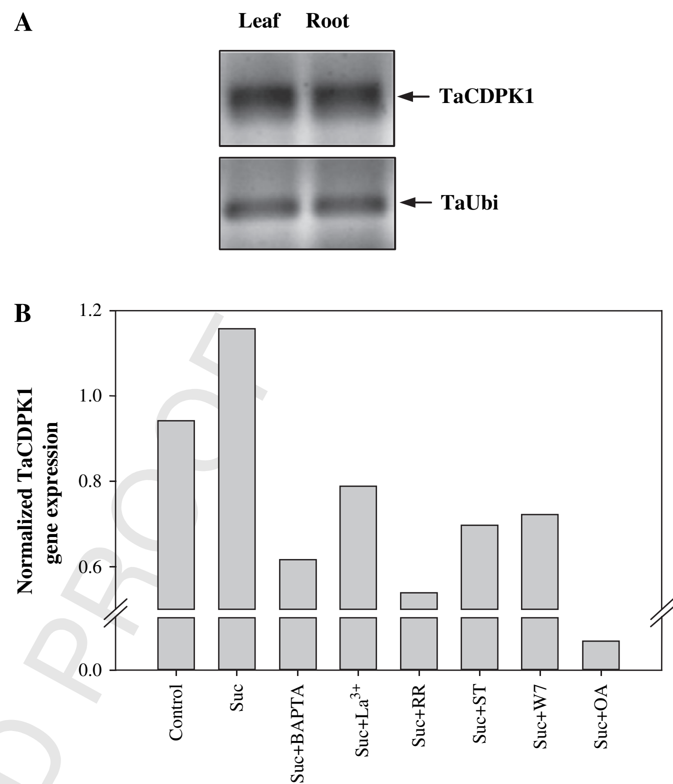

Fig. 4. TaCDPK1 gene expression analysis (A) Semi-quantitative gene expression analysis of TaCDPK1 in excised leaves and roots of wheat seedlings as compared to Ubi (control). Ethidium bromide-stained gels after RT-PCR amplification using TaCDPK1 specific primers. (B) Quantitative RT-PCR analysis of TaCDPK1 gene expression in excised leaves of wheat fed with water as control, 200 mM sucrose (Suc), or 200 mM Suc with the addition of either 5 mM BAPTA or 10 mM La^{3+} or 50 μM RR or 200 μM W7 or 2 μM ST or 1 μM OA. The copy numbers of TaCDPK1 were normalized with Ubi levels in the same sample. The experiments were repeated at least 2 more times with similar results.

4. Discussion

We are interested in the identification of components of the Suc induced signaling pathway that control the expression of Suc responsive genes. In the present study, using a RT-PCR based approach, we cloned and characterized a Suc inducible CDPK cDNA (designated as TaCDPK1) from wheat. The deduced amino-acid sequence of the cloned TaCDPK1 contains a catalytic Ser/Thr kinase domain in the N-terminus and four putative Ca^{2+} binding EF hands in the C-terminus of TaCDPK1, distinctive feature of CDPKs [6]. The consensus motif for myristoylation at the N-terminal of the putative TaCDPK1 suggests a possible membrane associated protein [12,19,20], while a rice isoform (OsCDPK14) lacking this motif has been localized in the cytoplasm [21]. Phylogenetic analysis indicates that TaCDPK1 protein belongs to the subgroup IIa of the CDPK family and that clustering of CDPK sequences is completely species independent and highly influenced by the sequence of the functional domains.

When heterologously expressed, the *E. coli* derived TaCDPK1 gene product showed binding to polyclonal antibodies raised against the soybean CDPK suggesting that the

UNCORRECTED PROOF

Please cite this article in press as: G. Martínez-Noël et al., Plant Physiol. Biochem. (2007), doi:10.1016/j.plaphy.2007.03.004

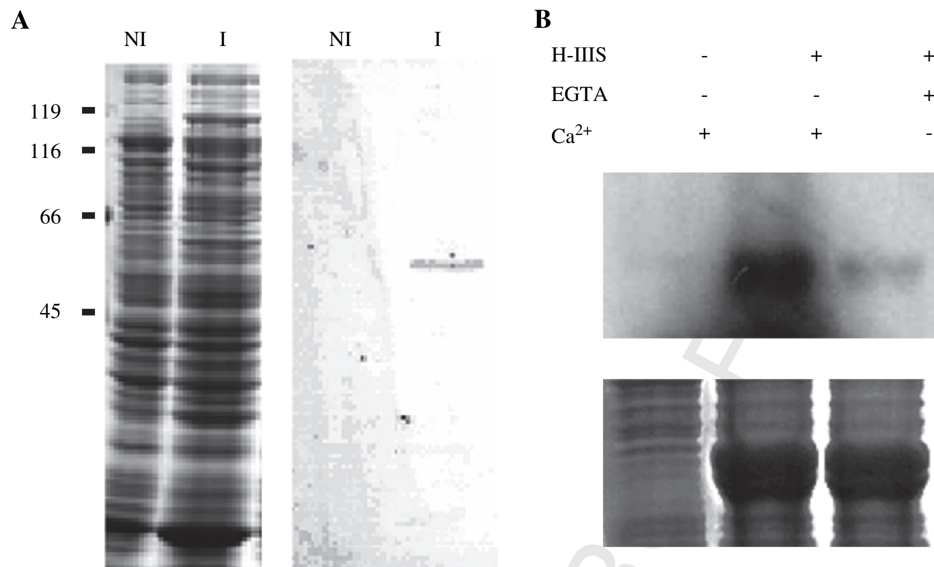

Fig. 5. Immunodetection and protein kinase activity of the recombinant *E. coli* derived TaCDPK1. (A) Protein extracts from bacteria transformed with a construct to express TaCDPK1 either not induced (NI) or induced (I) for transgene expression were resolved on 10% SDS-PAGE and stained with Coomassie blue (left) or transfer to a membrane that was revealed with polyclonal antibodies raised against the soybean CDPK (right). The position of the molecular mass markers are indicated in kDa. (B) Proteins extracts from transformed and induced bacteria were incubated with [γ-^{32}P]ATP at room temperature for 30 min, in the presence or absence of histone III-S (exogenous substrate), EGTA (Ca^{2+} chelator) and Ca^{2+}. The reactions products were subjected to 10% SDS-PAGE, stained with Coomassie blue (bottom panel) and then subjected to autoradiography (top panel).

proteins from these two plants are very similar. The recombinant enzyme was able to efficiently catalyze the phosphorylation of histone III-S, a commonly used substrate for protein kinase assays. However, the absence of Ca^{2+} considerably diminished the phosphorylation activity of the recombinant enzyme indicating that TaCDPK1 activity is clearly dependent on this ion. In vivo, apart from Ca^{2+}, other signaling molecules like phospholipds may further influence activity of plant CDPKs [22]. Autophosphorylation of recombinant TaCDPK1 was also obtained (data not shown), as it has been observed in almost all CDPK studied [23].

CDPKs fulfill a vital role in Ca^{2+} mediated signal transduction events [9,10,24]. Diverse stimuli, including light, phytohormones, fungal elicitors, drought, phospholipids, low temperature, salt stress and sugars, have been reported to alter Ca^{2+} levels in the cell [5,25]. Sugars are also known to induce an increase in cytosolic Ca^{2+} content in tobacco leaves [26–28]. Using a fluorescent probe (Fluo3-AM), we found that cytosolic Ca^{2+} levels rapidly increase during Suc treatment of wheat leaves and roots [29]. By binding Ca^{2+}, CDPKs can potentially decode the Ca^{2+} mediated sugar signal and subsequently activate downstream targets through their kinase activity [9]. Therefore, for an efficient relay of the sugar signal, appropriate CDPK protein levels are required and this is probably ensured by an upregulation of gene expression. The induction of sweet potato and potato CDPKs (StCDPK1) have also been reported by high sugar concentration [30,31] and we found that Suc stimulates CDPK gene expression in wheat leaves in a time dependent manner (data not shown). Both StCDPK1 and TaCDPK1 share a high sequence identity and both genes may be regulated similarly by sugar enhanced Ca^{2+} levels. The expression of other genes highly similar to TaCDPK1 is known to be inducible by hormones and in response to abiotic/biotic stresses (wounding, temperature shock, fungic toxins, etc.) [32–34].

If the association of Ca^{2+} with CDPK is inhibited either by withdrawal of Ca^{2+} from the system using BAPTA or blocking Ca^{2+} transport (La^{3+} and RR) or by antagonists of CDPKs calmodulin domain (W7), then, Suc is ineffective in the induction of FT gene expression [15]. In this study, we found that these inhibitors also prevented the upregulation of TaCDPK1 gene expression by Suc, indicating that the level of Ca^{2+} or its interaction with CDPK could have an influence on TaCDPK1 transcription itself.

Using the inhibitor OA, we have demonstrated that protein phosphatases (PPs) also participate in the Suc signal transduction pathway responsible for the regulation of the expression of FT genes [15]. Our results indicate that PPs mediate the Suc induction of TaCDPK1 gene since OA completely blocked this effect. Thus, we propose that PPs may be upstream to TaCDPK1 in the Suc signaling pathway. We recently found that PP2A could be involved in the sucrose uptake by the cells in wheat leaves (unpublished results). TaCDPK1 may also be involved in the signaling pathways related to house-keeping activities as suggested by a basal level of TaCDPK1 gene expression in freshly harvested root and leaf tissue.

With respect to Suc induced fructan accumulation, our understanding of the Suc sensing and the subsequent information transmission events leading to gene expression are gradually improving. A threshold Suc concentration, specific for mesophyll and parenchymatous bundle sheath cells [35], is probably sensed in a hexokinase independent manner [36] and triggers a signal transduction pathway that sequentially involves PPs, Ca^{2+}, CDPKs and along with other components

[15]. The signaling events culminate in the transcriptional activation of FT genes through Suc responsive elements located in their promoter region [37]. The activity of the 6-SFT promoter in transgenic *Arabidopsis* plants was found to be drastically inhibited by BAPTA and ST- a Ser/Thr protein kinase inhibitor (unpublished results). This suggests that both Ca^{2+} and CDPK may be general components of Suc signaling pathways in plants and not just specific to the regulation of fructan synthesis since *Arabidopsis* lacks fructan metabolism. The newly cloned TaCDPK1 will help further studies that aim to clarify the Suc signaling pathway.

Acknowledgments

This work was partially supported by the Fundación Antorchas, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica, Universidad Nacional de Mar del Plata (UNMdP) and Fundación para Investigaciones Biológicas Aplicadas (FIBA). We thank Dr. Alice H. Harmon (University of Florida, Gainesville) for generously providing the polyclonal antibody against soybean CDPK and Dr. Graciela Salerno for critical reading of the manuscript. G.M.N. is a fellow of CONICET, H.G.P. is a Career Investigator of CONICET.

References

[1] J.M. Stone, J.C. Walker, Plant protein-kinase families and signal-transduction, Plant Physiol. 108 (1995) 451–457.
[2] S.K. Sopory, M. Munshi, Protein kinases and phosphatases and their role in cellular signaling in plants, Crit. Rev. Plant Sci. 17 (1998) 245–318.
[3] T. Hunter, A thousand and one protein kinases, Cell 50 (1987) 823–829.
[4] D.M. Roberts, A.C. Harmon, Calcium-modulated proteins-targets of intracellular calcium signals in higher-plants, Annu. Rev. Plant Phys. 43 (1992) 375–414.
[5] D. Sanders, J. Pelloux, C. Brownlee, J.F. Harper, Calcium at the crossroads of signaling, Plant Cell 14 (2002) S401–S417.
[6] A.C. Harmon, M. Gribskov, E. Gubrium, J.F. Harper, The CDPK superfamily of protein kinases, New Phytol. 151 (2001) 175–183.
[7] S.H. Cheng, M.R. Willmann, H.C. Chen, J. Sheen, Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family, Plant Physiol. 129 (2002) 469–485.
[8] E.M. Hrabak, C.W. Chan, M. Gribskov, J.F. Harper, J.H. Choi, N. Halford, J. Kudla, S. Luan, H.G. Nimmo, M.R. Sussman, M. Thomas, K. Walker-Simmons, J.K. Zhu, A.C. Harmon, The Arabidopsis CDPK-SnRK superfamily of protein kinases, Plant Physiol. 132 (2003) 666–680.
[9] J.E. Harper, G. Breton, A. Harmon, Decoding Ca^{2+} signals through plant protein kinases, Annu. Rev. Plant Biol. 55 (2004) 263–288.
[10] A.A. Ludwig, T. Romeis, J.D.G. Jones, CDPK-mediated signalling pathways: specificity and cross-talk, J. Exp. Bot. 55 (2004) 181–188.
[11] A.C. Harmon, B.C. Yoo, C. McCaffery, Pseudosubstrate inhibition of Cdpk, a protein-kinase with a calmodulin-like domain, Biochemistry-Us 33 (1994) 7278–7287.
[12] F. Rutschmann, U. Stalder, M. Piotrowski, C. Oecking, A. Schaller, LeCPK1, a calcium-dependent protein kinase from tomato. Plasma membrane targeting and biochemical characterization, Plant Physiol. 129 (2002) 156–168.
[13] N.G. Halford, M.J. Paul, Carbon metabolite sensing and signalling, Plant Biotechnol. J. 1 (2003) 381–398.
[14] F. Rolland, E. Baena-Gonzalez, J. Sheen, Sugar sensing and signaling in plants: conserved and novel mechanisms, Annu. Rev. Plant Biol. 57 (2006) 675–709.
[15] G.M. Martínez-Noël, J.A. Tognetti, H.G. Pontis, Protein kinase and phosphatase activities are involved in fructan synthesis initiation mediated by sugars, Planta 213 (2001) 640–646.
[16] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[17] Y. Saijo, S. Hata, J. Sheen, K. Izui, cDNA cloning and prokaryotic expression of maize calcium-dependent protein kinases, Biochim. Biophys. Acta 1350 (1997) 109–114.
[18] T. Asano, N. Tanaka, G. Yang, N. Hayashi, S. Komatsu, Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: comprehensive analysis of the CDPKs gene family in rice, Plant Cell Physiol. 46 (2005) 356–366.
[19] M.L. Martin, L. Busconi, Membrane localization of a rice calcium-dependent protein kinase (CDPK) is mediated by myristoylation and palmitoylation, Plant J. 24 (2000) 429–435.
[20] M. Ellard-Ivey, R.B. Hopkins, T.J. White, T.L. Lomax, Cloning, expression and N-terminal myristoylation of CpCPK1, a calcium-dependent protein kinase from zucchini (*Cucurbita pepo* L.), Plant Mol. Biol. 39 (1999) 199–208.
[21] T. Zhang, Q. Wang, X. Chen, C. Tian, X. Wang, T. Xing, Y. Li, Y. Wang, Cloning and biochemical properties of CDPK gene OsCDPK14 from rice, J. Plant Physiol. 162 (2005) 1149–1159.
[22] J. Szczegielniak, M. Klimecka, A. Liwosz, A. Ciesielski, S. Kaczanowski, G. Dobrowolska, A.C. Harmon, G. Muszynska, A wound-responsive and phospholipid-regulated maize calcium-dependent protein kinase, Plant Physiol. 139 (2005) 1970–1983.
[23] A.D. Hegeman, M. Rodriguez, B. Woo Han, Y. Uno, G.N. Phillips Jr., E.M. Hrabak, J.C. Cushman, J.F. Harper, A.C. Harmon, M.R. Sussman, A phyloproteomic characterization of *in vitro* autophosphorylation in calcium-dependent protein kinases, Proteomics 6 (2006) 3649–3664.
[24] A.C. Harmon, M. Gribskov, J.F. Harper, CDPKs-a kinase for every Ca^{2+} signal? Trends Plant Sci. 5 (2000) 154–159.
[25] S. Pandey, S.B. Tiwari, K.C. Upadhyaya, S.K. Sopory, Calcium signaling: linking environmental signals to cellular functions, Crit. Rev. Plant Sci. 19 (2000) 291–318.
[26] M.A. Ohto, K. Hayashi, M. Isobe, K. Nakamura, Involvement of Ca^{2+} signaling in the sugar-inducible expression of genes-coding for sporamin and beta-amylase of sweet-potato, Plant J. 7 (1995) 297–307.
[27] X. Vitrac, F. Larronde, S. Krisa, A. Decendit, G. Deffieux, J.M. Merillon, Sugar sensing and Ca^{2+}-calmodulin requirement in *Vitis vinifera* cells producing anthocyanins, Phytochemistry 53 (2000) 659–665.
[28] T. Furuichi, I.C. Mori, K. Takahashi, S. Muto, Sugar-induced increase in cytosolic Ca^{2+} in *Arabidopsis thaliana* whole plants, Plant Cell Physiol. 42 (2001) 1149–1155.
[29] G. Martínez-Noël, J. Tognetti, V. Nagaraj, A. Wiemken, H. Pontis, Calcium is essential for fructan synthesis induction mediated by sucrose in wheat, Planta 225 (2006) 183–191.
[30] M. Otho, K. Nakamura, Sugar-induced increase of calcium-dependent protein kinases associated with the plasma membrane in leaf tissues of tobacco, Plant Physiol. 109 (1995) 973–981.
[31] M. Raices, G.C. Macintosh, R.M. Ulloa, P.R. Gargantini, N.F. Vozza, M.T. Tellez-Iñon, Sucrose increases calcium-dependent protein kinase and phosphatase activities in potato plants, Cell Mol. Biol. 49 (2003) 959–964.
[32] G.M. Yoon, H.S. Cho, H.J. Ha, J.R. Liu, H.S.P. Lee, Characterization of NtCDPK1, a calcium-dependent protein kinase gene in *Nicotiana tabacum*, and the activity of its encoded protein, Plant Mol. Biol. 39 (1999) 991–1001.
[33] S. Saijo, S. Hata, J. Kyozuka, K. Shimamoto, K. Izui, Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants, Plant J. 23 (2000) 319–327.
[34] S. Davletova, T. Meszaros, P. Miskolczi, A. Oberschall, K. Torok, Z. Magyar, D. Dudits, M. Deak, Auxin and heat shock activation

UNCORRECTED PROOF

Please cite this article in press as: G. Martínez-Noël et al., Plant Physiol. Biochem. (2007), doi:10.1016/j.plaphy.2007.03.004

of a novel member of the calmodulin like domain protein kinase gene family in cultured alfalfa cells, J. Exp. Bot. 52 (2001) 215–221.

[35] C. Pollock, J. Farrar, D. Tomos, J. Gallagher, C.G. Lu, O. Koroleva, Balancing supply and demand: the spatial regulation of carbon metabolism in grass and cereal leaves, J. Exp. Bot. 54 (2003) 489–494.

[36] J. Muller, R.A. Aeschbacher, N. Sprenger, T. Boller, A. Wiemken, Disaccharide-mediated regulation of sucrose: fructan-6-fructosyltransferase, a key enzyme of fructan synthesis in barley leaves, Plant Physiol. 123 (2000) 265–273.

[37] V.J. Nagaraj, R. Riedl, T. Boller, A. Wiemken, A.D. Meyer, Light and sugar regulation of the barley sucrose: fructan 6-fructosyltransferase promoter, J. Plant Physiol. 158 (2001) 1601–1607.

[38] S. Kumar, K. Tamura, M. Nei, MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment, Brief Bioinform 5 (2004) 150–163.

UNCORRECTED PROOF

Please cite this article in press as: G. Martínez-Noël et al., Plant Physiol. Biochem. (2007), doi:10.1016/j.plaphy.2007.03.004