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Characterization of CDKA gene expressed during *in vitro* regeneration from pepper (*Capsicum annuum* L.) cotyledon explants

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Abstract

Adventitious bud-like structures were directly regenerated from pepper (*Capsicum annuum* L. cv. Baklouti) cotyledon explants taken from 14 days-old seedlings and cultivated on MS medium supplemented with 5.7 µM IAA and 8.8 µM BAP. Histological and molecular studies were performed at different stages of the *in vitro* culture process in order to follow the regeneration pathway. Histological study revealed that a cell dedifferentiation process took place at the periphery of the excised petiolar side of cotyledon after 4 days of culture. It led to the formation of teratological protuberances resulting in the development of disorganized apical shoot meristem. In order to follow the regeneration process at the molecular level, the study was based on CDKA gene expression analysis. This gene was chosen because of its major role in the regulation of eukaryotic cell cycle. The CDKA mRNA transcription rate study revealed a steady-state transcript level during all the developmental phases except at the dedifferentiation step where an increase was noticed. The Western blot analysis showed that CDKA protein was particularly expressed in initial cotyledons of 14 days-old seedlings, declined until dedifferentiation stage and tended to reincrease during the subsequent stages. All these results suggest that CDKA expression may be linked to dedifferentiation during adventitious organogenesis in pepper tissues cultivated *in vitro*. It can therefore be used as a molecular marker for *in vitro* regeneration in this recalcitrant species.

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Introduction

Chili pepper (*Capsicum annuum* L.) is a very important crop grown worldwide. Unlike other Solanaceous species, *Capsicum annuum* L. is considered as a recalcitrant species with regard to its capacity for *in vitro* regeneration. In general, induced buds failed to elongate or produce stunted stems (Arroyo and Revilla, 1991, Steinitz *et al.*, 2003, Kothari *et al.*, 2010). Several attempts were tried to optimize culture conditions, but only few explanation were given to this rebel behaviour. Histological investigations revealed that the scare plant regeneration seems to be a consequence of infrequent differentiation of normal apical shoot meristems and not due to a defect in shoot development from available meristems **(**Wolf *et al.*, 2001, Mezghani *et al.*, 2007).

Plant morphogenesis involves close control and coordination of proliferative activity through regulation of the cell cycle in meristematic tissues (Sugiyama, 1999; Planchais *et al.*, 2000). Artificial initiation and maintenance of cell division is a prerequisite for generation and establishment of the dedifferentiated targeted meristematic cell. The major regulators of eukaryotic cell cycle are Cyclin-Dependent Kinases (CDKs) and their regulatory pattern cyclins (De Veylder *et al.,* 2003, Dewitte and Murray, 2003). In the model plant Arabidopsis, 12 CDKs have been found, subdivided into six distinct classes from CDKA to CDKF (Vandepoele *et al.,* 2002). The CDKA is the most abundant class comprising CDKs that are most closely related to the prototypical CDKs from yeast CDC2/CDC28 and animal CDC2/CDK1 and CDK2. It contains an evolutionary conserved 16 amino acid sequence called PSTAIRE motif which is essential for cyclin binding (Morgan, 1997, Mironov *et al.,* 1999). CDKA expression was not found restricted to dividing cells, but was also observed at a relatively high level even in non-dividing differentiated plant cells, such as those in young leaves, marking competence for cell division (Hemerly *et al.*, 1993). During direct somatic embryogenesis in *Cocos nucifera*, a progressive decrease was observed in CnCDKA expression with the lowest expression in germinated somatic embryos. Whereas a steady increased was observed during embryogenic callus formation phase when embryogenic competence is attained (Montero-Cortes *et al.*, 2010). In *Prunus incise*, the expression profile of PiCDKA showed two main phases followed by a final decline wich was correlated with the progressive reduction of cell divisions (Ben Mahmoud *et al.*, 2013).

The objective of the present study was to isolate CDKA homolog from pepper genome and to study its expression during the process of adventitious organogenesis on cotyledons explants cultivated *in vitro* in order to find out eventual relationships between the different morpho-histolological reactions observed and the expression of pepper Capan;CDKA.

Materials and methods

Plant material and culture conditions

Experiments were carried out with cotyledon explants of hot pepper cv. Baklouti (a local cultivar well appreciated by Tunisian consumers). Seeds were sterilised with 20% commercial bleach for 10 min and then repeatedly washed with sterile distilled water. They were aseptically sown on MS (Murashige and Skoog, 1962) basal medium (pH 5,8) solidified with 0,8% agar and incubated in a growth room at 25 ± 2 °C during 5 days in the dark (until radicle emergency) and then for 9 days under a 16 h daily photoperiod (40 µmol. m^{-2}. s^{-1}). Cotyledons excised from 14 days-old seedlings were transferred in culture medium composed by basal MS medium supplemented with 5.7 µM IAA and 8.8 µM BAP. Cultures were incubated in the growth chamber and morphogenetic responses were recorded after three weeks of culture.

Histological analysis

Histological investigations were carried out on cotyledons either during germination process from 5 days and 14 days-old seedlings or after transfer in culture medium at different stages of the culture. Four Samples per harvest date were fixed in FAA (formalin, acetic acid, absolute alcohol: 10, 5, 85 v/v) for 18 h. Tissues were then dehydrated in graded

series of ethanol (50°, 70°, 96° and 100°) and embedded in paraffin. Serial sections of 10 µm were made using a RM2125RT microtome and slides containing the sections were passed through a series of deparaffinazing and hydrating solutions, stained with safranin (RAL Reactifs) and hematoxylin (Panreac Quimica SA) and observed under a photonic microscope (Leica DMLB2).

Partial cloning of pepper CDKA cDNA

Total RNA from cotyledons of 5 days-old seedlings was extracted using RNeasy® Plant Mini Kit (50) (Qiagen) according to the manufacturer's instructions.

To synthesise the cDNA first-strand, 2 µg of RNA were incubated for 90 min at 42 °C in the presence of 120 pmol of oligodT primer and 25 units of Stratascript reverse transcriptase (Stratagene) in a 20 µl mixture reaction. An aliquot of 5 µl was then subjected to polymerase chain reaction (PCR) in the presence of specific primers: CaCdkAf 5'-TCTAAggatccCCGTGTTGAAAAACGTTTT-3' and CaCdkAr 5'-TAATCAgagctcTCAGTGCGTCCTTGAGGGAGC-3'.

Their sequences were deduced from an EST submitted in The Gene Index (TGI) data base (http://compbio.dfci.harvard.edu/tgi; accession no. TC5772) that has shown a high degree (88%) of homology with tomato CDKA;1 (GenBank accession no. Y17225). This pepper EST was isolated from flower buds after 10 weeks of germination in relation to hypersensitive response against pathogen. Small letters represent *Bam*HI and *Sac*I restriction sites included to facilitate the subsequent cloning.

The amplification procedure was performed using 1 U of Taq DNA polymerase (Promega) in the presence of 20 pmol of each primer and 200 µM of dNTP mixture in a final volume of 20 µl. An initial denaturation step of 5 min at 94 °C was followed by the reaction programme: 30 s at 94°C, 30 s at 56°C and 1 min at 72 °C for 32 cycles and a final extension of 7 min at 72 °C. The amplification product was purified by DNA Gel Extraction kit (Millipore) and subcloned into pBluescript II KS$^+$ vector (Stratagene). The resulting plasmids were subjected to DNA sequencing.

Semi-quantitative RT-PCR analysis

The total RNA was extracted as described before (section 2. 2) from cotyledons of 5 and 14 days-old seedlings and from cotyledons after 2, 4, 8 and 12 days of culture. At day 15 of culture, well developed bud-like structures appeared and served for the RNA extraction.

The RT-PCR was performed as described previously. As a control for RT-PCR expression pattern, a 1049-pb cDNA fragment for actin 8 gene was amplified using 2 µl of the RT reaction and the following set of primers: CaActF 5'-GAGAGACTAAGCACATCATCTCCG-3' and CaActR 5'-AATCCAGACACTGTATTTTCTCTG-3'. The amount of first-strand cDNA and the number of cycles used allowed the reaction to be in the linear range of PCR amplification. Contamination by genomic DNA of the total RNA was determined by a PCR assay where the reverse transcription was omitted.

Ten microliters of the PCR product was subjected to electrophoresis on 1% agarose gel and stained with ethidium bromide. The software AlphaImager for Windows was used to analyse the expression levels of CDKA for each stage with actin 8 as a control. Each PCR and electrophoresis procedure was repeated twice. The average of CDKA and actin 8 expression was first calculated for each sample before determination of the CDKA/actin 8 ratio. The CDKA expression ratios were then compared between the different stages.

Western blot analysis

The same cotyledon development stages used for RNA extraction were chosen in this study. Cotyledons were ground to a fine powder in liquid nitrogen and thawed in 3 volumes of extraction buffer consisting of 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 2 mM PMSF. The cell debris was discarded after a 15 min centrifugation at 12500g and 4 °C.

After quantification by Bradford (1976) method using Bovine Serum Albumin as standard, total proteins (20 µg of each stage) were separated in 15 % SDS-polyacrylamide gel (Laemmli 1970) and transferred to nitrocellulose (Hybond C; Amersham) membrane.

Western blot was performed according to Harlow and Lane (1988) using an anti-PSTAIRE monoclonal antibody (Sigma; 1:4000 dilution). ECL western blot detection kit (Amersham) was used to reveal hybridization pattern using peroxidase conjugated anti-mouse Immunoglobulin (Kirkegaard & Perry Laboratories, 1:2500 dilution).

Results

Cotyledon anatomy during germination

The fundamental changes accompanying the germination process were investigated as a prerequisite to understand the events occurring during *in vitro* regeneration. The analysis of the concomitant modifications that affect cotyledon tissue at the histological level was performed. It concerned cotyledons from 5 days-old seedlings corresponding to radicle emergency (Fig. 1 a) and fully developed cotyledons from 14 days-old seedlings (Fig. 1 c). The last ones were used as starting material for *in vitro* regeneration.

Anatomical observation showed that cells of first cotyledon category had a meristematic feature i. e. small, densely stained with big nucleus (Fig. 1 b); whereas cells of the second category were parenchymatous and differentiated (Fig. 1 d).

Morpho-histological changes on cotyledons during in vitro regeneration

The hormonal treatment of cotyledon explants led to an increase in their size and the petiolar side became larger and whitish after 4 days of culture (Fig. 2 a). These morphological changes corresponded to the setting of a dedifferentiation process giving rise to smaller cells with clear nucleus on the epidermal and subepidermal tissue of the petiole (Fig. 2 b). This cell activity led to the formation teratological protuberances covering the cut end of the explant after nearly 12 days of culture (Fig. 2 c, d). Further development of these structures resulted in the formation of bud-like structures (Fig. 2 e) with disorganized apices and leaf primordia (Fig. 2 f) after 15 days.

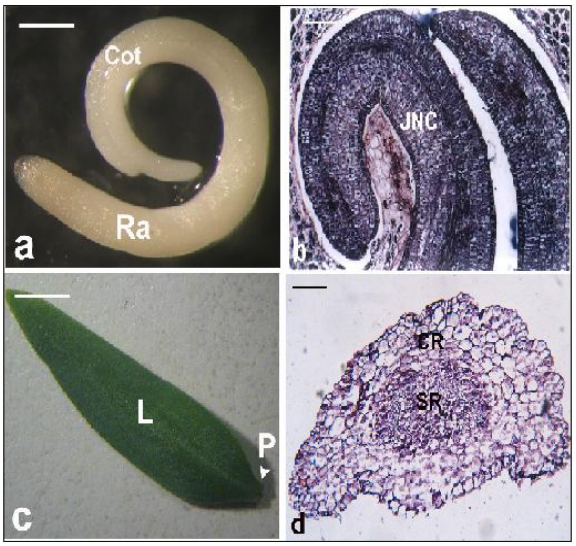

Fig. 1. Morphological and anatomical state of pepper (*C. annuum* cv. Baklouti) cotyledons during germination process. a. Radicle (Ra) emergency stage after 5 days. Cot: cotylédon (Bar = 0.4 mm). b. Histological aspect of cotyledon after 5 days. JNC: juvenile cells with clear nucleus (Bar = 80 µm) c. Fully developed cotyledon after 14 days (Bar = 0.63 mm). P: petiole; L: Limb. d. Anatomy of the petiole side after 14 days. SR: stellar region composed by vascular bundles surrounding a pith region of parenchyma cells; CR: Cortical region composed by a single layered epidermis; 1-2 layers of collenchyma; 5-6 layers of parenchyma (Bar = 80 µm).

Partial cloning of pepper CDKA cDNA (Capan;CDKA;1)

For understanding the process of *in vitro* regeneration from pepper cotyledon explant at the molecular level, CDKA gene expression was investigated. As a first step, isolation and analysis of a pepper CDKA gene fragment were performed in order to have a specific marker for the gene. We used the RT-PCR strategy to isolate a partial CDKA cDNA from 5 days-old seedlings as described in materials and methods. A single DNA band of 700 bp was obtained under these conditions. This cDNA was then cloned in pSK^+ and sequenced. The results showed that the amplified pepper cDNA (Capan;CDKA;1) harbours a

570-bp long open reading frame encoding a 190-amino acid product. It was deposited in GenBank under the accession no. EL515581.

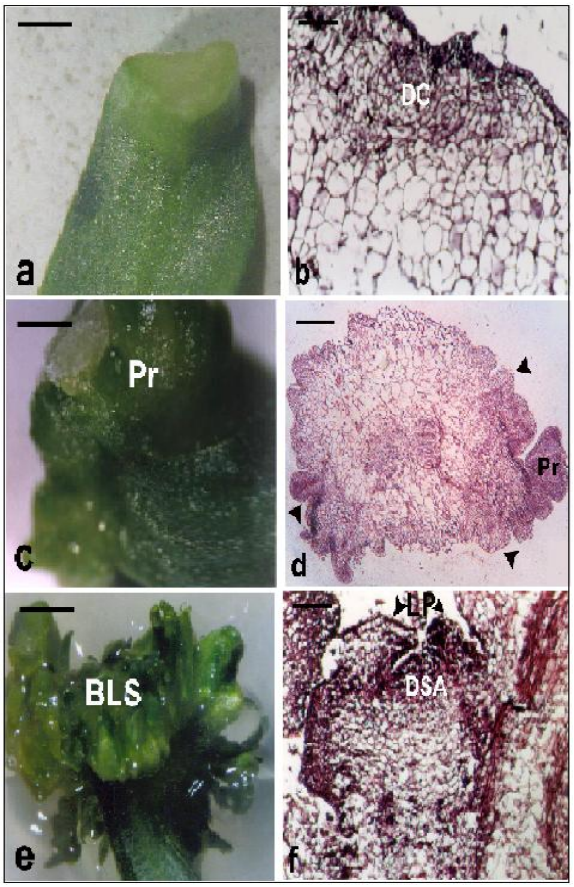

Fig. 2. Morpho-histological changes occurring on the petiolar side of pepper (*C. annuum* cv. Baklouti) cotyledon explants cultivated on MS medium supplemented with 5.7 µM and 8.8 µM BAP a. General view of cotyledon after 4 days of culture (Bar = 0.63 mm). b. Longitudinal section showing the presence of dedifferentiating cells (DC) in the epidermal and subepidermal tissues after 4 days of culture (Bar = 10 µm). c. General view of protuberances (Pr, indicated by arrows) after 12 days of culture (Bar = 0.4 mm). d. Transverse section showing numerous protuberances (Pr) around the cut end of the explant after 12 days of culture (Bar = 80 µm). e. General view of adventitious bud-like structures (BLS) after 15 days of culture (Bar = 0.63 mm). f. Longitudinal section showing a disorganized shoot apex (DSA) after 15 days of culture; LP: Leaf primordia (Bar = 40 µm).

The sequence analysis based on BLAST search revealed that this cDNA displayed 89% identity with cyclin dependent kinase CDKA;1 from tomato (*Lycopersicon esculentum*, Y17225); 88% with CDKA;4 from tobacco (*Nicotiana tabacum*, AF289467) and 80% with CDKA;1 from maize (*Zea mays*, A40444) and *Arabidopsis* (*Arabidopsis thaliana*, M59198). The phylogenetic study showed that Capan;CDKA;1 is closely related to CDKA of solanaceous species including tomato and tobacco (Fig. 3). The alignment of these sequences with Clustal W (1.83) program (Fig. 4) showed that Capan;CDKA;1 contains functionally important regions characteristic of CDK such as two highly conserved HRDLKPQNLLI (amino acids 20-30) and WYRAPEILL (amino acids 64-72) domains (Miao *et al.*, 1993, Lindroth *et al.*, 2001) in addition to the T loop area (residues 147-172) centred around Thr-161 (at position 57 in Capan;CDKA;1) and the T-loop flanking Asp-146 (at position 42 in Capan;CDKA;1). The phosphorylation of CDKA at Thr-161 residue stabilizes the cyclin binding and Thr-161 residue is involved in the positioning of bound ATP required for kinase activity (Joubès *et al.*, 1999).

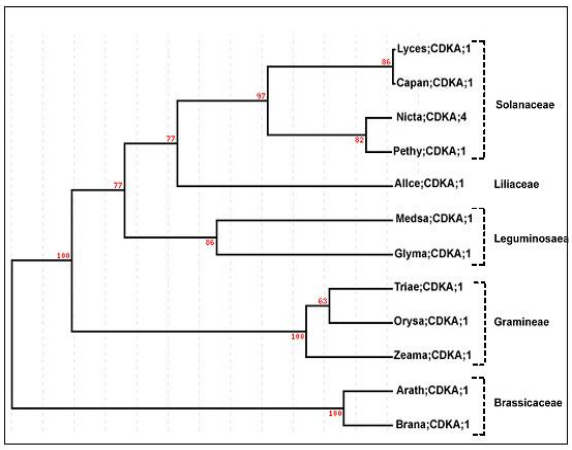

Fig. 3. Relationship between Capan;CDKA;1 and other plant CDKA proteins. CDKA sequences are indicated according to the new nomenclature proposed by Joubès *et al.* (2000). The sequences are Lyces;CDKA;1 (*Lycopersicon esculentum*, Y17225) ; Nictab;CDKA;4 (*Nicotiana tabacum*, AF289467); Pethy;CDKA;1 (*Petunia hybrida*, Y13646) ; Allcea;CDKA;1 (*Allium cepa*, AB006033) ; Medsa;CDKA;1 (*Medicago sativa*, M58365) ; Glyma;CDKA;1 (*Glycine Max*, M93140) ; Triae;CDKA;1 (*Triticum aestivum*, U23409) ; Orysa;CDKA;1 (*Orysa sativa*, X60374) ;

Zeama;CDKA;1 (*Zea mays*, A40444) ; Arath;CDKA;1 (*Arabidopsis thaliana*, M59198) and Brana;CDKA;1 (*Brassica napus*, U18365). The phylogenetic tree was performed using Phylip program (http://www.genebee.msu.su/services/phtree_reduced.html). Numbers shown represent bootstrap values exceeding 50 out of 100 replicates.

Analysis of differential expression of Capan;CDKA;1 during adventitious organogenesis

In order to study the transcriptional regulation of Capan;CDKA;1 gene expression during the first two weeks of culture, the RNA preparations were analyzed by RT-PCR. Since it was not possible to selectively isolate the reactive cells from the explants, only the proximal side of cotyledons that was the most responsive, was used in order to minimise the dilution effect by surrounding tissue. The data (Fig. 5 a, b) showed that Capan;CDKA;1 mRNA was present in similar amounts in all the developmental stages but it seemed to be particularly accumulated at day 4 of culture which corresponded to the cell dedifferentiation phase.

Western blot analysis using anti-PSTAIR antibody revealed the presence of two bands: the predicted 34 kDa band corresponding to p34cdc2 protein and an additional major one of higher molecular weight (approximately 36kDa). These proteins seemed to be highly expressed in cotyledons of 5 days-old seedlings. They declined until dedifferentiation stage and tended to reincrease during the subsequent stages (Fig. 6).

Discussion

The process of *in vitro* plant regeneration via adventitious organogenesis in pepper (*Capsicum annuum* L.) was widely discussed in the literature. Besides the low efficiency of plant regeneration that has often been described (Liu *et al.,* 1990, Ochoa-Alejo and Ireta-Moreno, 1990, Mathew, 2002, Mok and Norzulaani, 2007) these reports didn't provide clear information about the fundamental mechanisms controlling the organogenetic process in this species. Among these mechanisms, we have been interested by anatomical and molecular ones.

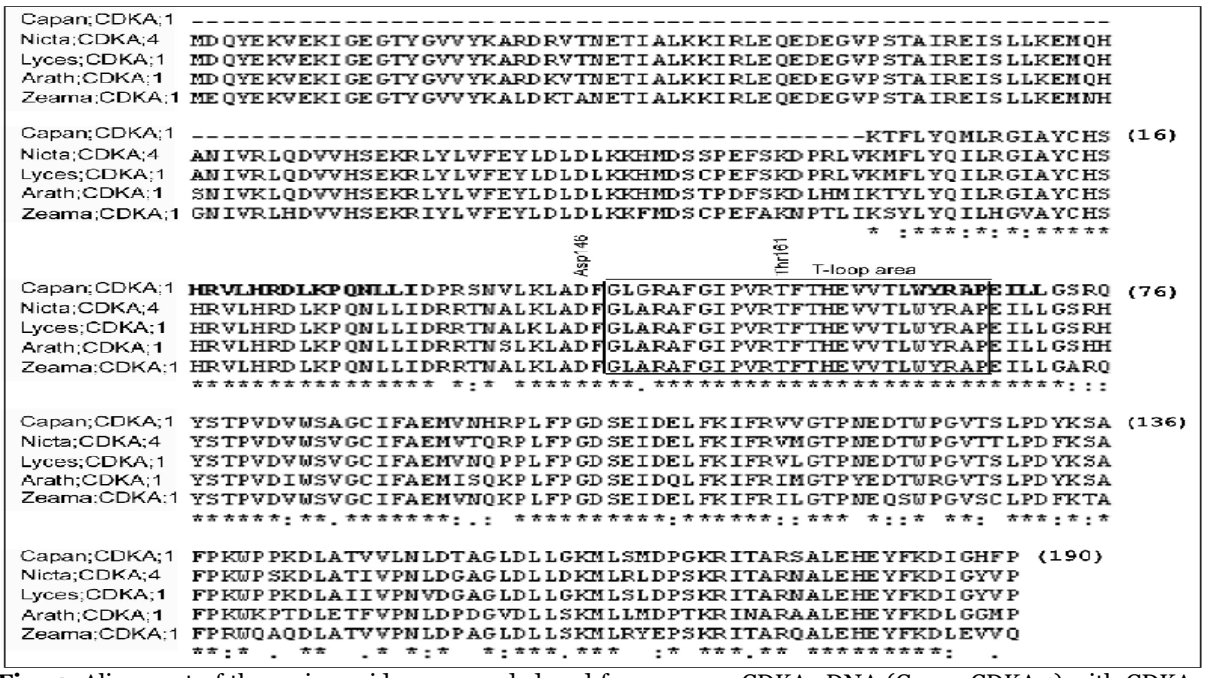

Fig. 4. Alignment of the amino acid sequence deduced from pepper CDKA cDNA (Capan;CDKA;1) with CDKA-related proteins from tomato (Lyces;CDKA;1), tobacco (Nicta;CDKA;4), maize (Zeama;CDKA;1) and *Arabidopsis* (Arath;CDKA;1). The sequences were aligned using CLUSTAL W (1.83) program. (*) denotes identical or conserved residues in all sequences in the alignment, (**:**) corresponds to conserved substitutions, (**.**) shows the semi-conserved substitutions.

Histological study provided evidence for a direct induction of adventitious bud-like structures from epidermal and sub-epidermal cotyledon tissues. It suggested that the low level of plant regeneration seemed to be the consequence of infrequent differentiation of normal apical shoot meristems rather than a defect in shoot development from available meristems (Mezghani *et al.,* 2007).

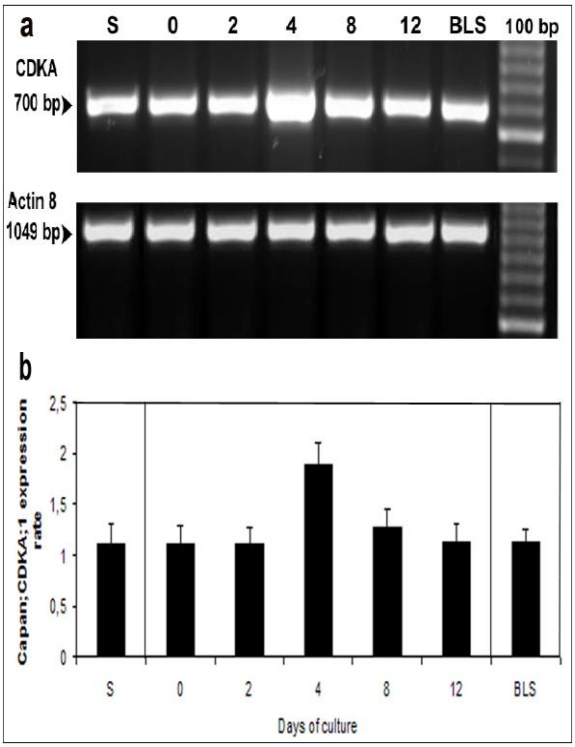

Fig. 5. Capan;CDKA;1 expression during adventitious regeneration. a. Electrophoresis on agarose gel (1%) of Capan;CDKA;1 RT-PCR products using Actin 8 as an internal control. b. Quantification of Capan;CDKA;1 transcripts using Alpha Imager software and the internal standard actin 8 signal as the denominator. Total RNA was isolated from cotyledons of 5 days-old seedlings (S), cotyledons before transfer on induction medium (0) and after respectively 2, 4, 8, 12 days of culture and from bud-like structures (BLS) after 15 days of culture; 100 bp: 100 bp DNA ladder (Invitrogen). Error bar represents standard deviation.

In order to further explain the morpho-histological results, a molecular approach based on CDKA gene expression was envisaged. Indeed, the corresponding protein is known to be involved in the regulation of cell division. The first step was therefore the cloning of a partial sequence of pepper CDKA cDNA. This sequence analysis showed that it encodes a putative protein displaying high similarity with CDKA proteins from solanaceous species e. g. tomato (Joubès *et al.,* 1999) and tobacco (Sorell *et al.,* 2001).

In a second step, RT-PCR and western blot analyses have been performed on cotyledon samples taken at different stages corresponding to particular histological events.

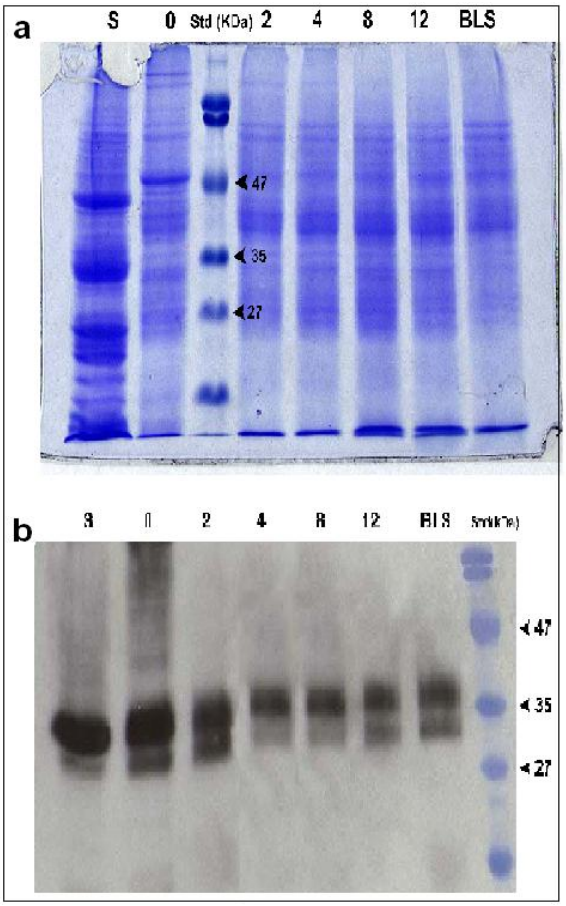

Fig. 6. Pepper CDKA protein expression during adventitious regeneration by western **b**lot analysis. a. Protein profile visualized by coomassie blue staining. b. Western blot performed using 20 µg of protein and an anti-PSTAIR monoclonal antibody (Sigma; 1:4000 dilution) and visualized with ECL chemiluminescent detection kit (Amersham). Total proteins were extracted from cotyledons of 5 d**a**ys-old seedlings (S), cotyledons before transfer on induction medium (0) and after respectively 2, 4, 8, 12, days of culture and from bud-like structures (BLS) after 15 days of culture ; Std: Low range prestained SDS-PAGE standard (Bio-Rad).

A confronting between the histological features and the pepper CDKA expression showed that the RNA was roughly correlated with the cell dedifferentiation phase which was characterized by a high division activity. Similar regulation pattern was also observed in *Arabidopsis thaliana* (Martinez *et al.,* 1992) and *Prinus incisa* (Ben Mahmoud *et al*., 2013). According to these data we can suggest that (i) under the influence of stimuli emitted by the *in vitro* culture conditions like phytohormones, wounding or environmental conditions, induction of CDKA expression may conduct appropriate cells in the way of dedifferentiation and division and as CDKA expression decreases, cells undergo differentiation or (ii) when cells are stimulated to divide by signals emitted by *in vitro* culture conditions, there is an induction of CDKA expression. However, when cells stop dividing and start to differentiate, CDKA transcription substantially decreases to rise again as cells reinitiate division (Hemerly *et al.,* 1993). In both cases, a close relationship between CDKA expression and a high mitotic activity can be established.

Similarly, Boucheron *et al.* (2002) reported that a larger amount of CDKA mRNA was detected in tobacco apical meristem and procambium as compared to cortical parenchyma, a tissue composed of terminally differentiated cells. Martinez *et al.* (1992) and Hemerly *et al.* (1993) suggested that in addition to the control of cell cycle progression, the CDKA expression could also have a role in cell totipotency i.e. the capacity of cells to re-enter the cell cycle. Lindroth *et al.* (2001) reported that expression of *Pinus contorta* CDKA (PcCDC2) during adventitious root development increased linearly during the first 12 days after an IBA treatment. The authors suggested that the PcCDC2 is most likely associated with competency for cell division rather than DNA replication since the CDKA mRNA accumulation was independent of H2A histone expression known to be associated with DNA replication in S phase of the cell cycle.

When the CDKA protein pattern was considered, the data showed that dedifferentiation seemed to be accompanied by a lower protein accumulation. However during the redifferentiation step the CDKA protein level tended to reincrease but it can not reach the initial rate. Different possibilities can explain this phenomenon: (i) CDKA proteins are present at high levels but not active or (ii) there is few CDKA protein amount despite the high transcription level of the gene. All these results confirm the complexity of CDKA activity control which is tightly linked to a variety of post-translational modifications including protein-protein interactions, reversible phosphorylation, and protein degradation (Lees, 1995, Joubès *et al.,* 1999, Mironov *et al.,* 1999, Potuschak and Doerner, 2001) in addition to a transcriptional control. However, Hemerly *et al.* (1993) suggested that the activation of CDKA expression is not only coupled with cell proliferation but may also precede it to allow cells to acquire the competence to divide.

Detection of two protein bands by western blot using a monoclonal anti-PSTAIR antibody can be the result of a post translational modification of the protein or may indicate that pepper possesses more than one CDK protein kinase belonging to CDKA class. This class comprises kinases most closely related to yeast (*Saccharomyces cerevisiae*, cdc28 and *Schizosaccharomyces pombe*, cdc2) and human (CDK1,-2,-3) which contain the typical canonical PSTAIRE amino-acid motif (Burssens *et al.,* 1998, Mironov *et al.,* 1999) and ensure distinct functions in the cell cycle from G1 to mitosis (Morgan, 1997). In addition to this group of CDKs, plants contain CDKB (PPTATLRE or PPTTLRE), CDKC (PITAIRE) and CDKE (SPTAIRE) among other CDKs (Inzé *et al.,* 1999, Vandepoele *et al.,* 2002, Gutierrez, 2005).

Conclusion

This study showed a positive correlation between CDKA gene expression and the proliferating state of pepper cotyledon tissue cultivated *in vitro*. Although CDKA expression seemed to be a useful molecular marker for the cell dedifferentiation phase, further studies are needed to characterize the expression of this gene and others critical ones in cell division and

shoot meristem development such as WUSCEL (WUS; Mayer *et al.,* 1998), KNOTTED1 (KN1; Vollbrecht *et al.,* 1991) and SHOOT MERISTEMLESS (STM; Long *et al.,* 1996). The characterization of the expression of these genes in combination with *in vitro* transgenic technology to overexpress or to inhibit these pivotal genes will likely be essential in understanding the process of plant morphogenesis especially in recalcitrant species such as pepper.

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Abbreviations: BAP: 6-benzylaminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid.