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Identification of programmed cell death related genes in bamboo☆,☆☆,★,★★

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ABSTRACT

The event of bamboo flowering and subsequent death of bamboo cells, a rare phenomenon is an interesting model to study gene expression/function in the context of the programmed cell death (PCD) in plant. To identify genes involved in autolytic cell death in bamboo (*Bambusa arundinacea*/*Bambusa bambos* Voss), a suppressive subtractive cDNA hybridization (SSH) was performed between cDNA isolated from control (healthy), as driver and test internodal tissue (45 days after setting of seeds), as tester. *In-silico* data revealed that 82% of total ESTs (231) were non-significant (unidentified proteins) while remaining ESTs were classified as protein with known/predicted function/s. Among these, net distribution and differential expression patterns of 11 important *B. arundinacea* PCD specific ESTs were studied using RNA slot-blot, qRT-PCR and semi-quantitative RT. *In-situ* localization of mRNA-transcripts for selected bamboo PCD-specific ESTs namely V2Ba48 (Aldehyde dehydrogenase 2) and V2Ba19 (Glycogen phosphorylase) were detected using digoxigenin-labeled corresponding anti-sense RNA probes employing Confocal Laser Scanning Microscope (CLSM). Differential expression-kinetics of the aforementioned genes were confirmed during the progress of PCD after setting of seeds. Global appearance of V2Ba48, V2Ba19, V2Ba95 (Ubiquitin thioesterase) and V2Ba89 (Nebulin isoform 2) genes were identified in monocot (*Oryza sativa*) and dicots (*Arabidopsis thaliana* and *Nicotiana tabacum*). This is the first report on systematic analysis of genes involved in death of bamboo cells that may provide critical information regarding key metabolic/regulatory genes involved in plant PCD.

1. Introduction

Programmed cell death (PCD) is an inter-linked cellular, nuclear and molecular event that ultimately leads to the controlled cell death in a pre-programmed fashion (Lockshin and Zakeri, 2004). It is crucial for the development of plant and animal (Bhalerao et al., 2003; Doyle et al., 2010; Greenberg, 1996; Harada et al., 2010; Jacobson et al., 1997; Kim et al., 2004; Qu et al., 2009); organ morphogenesis (Fekete et al., 1997; Tsujimoto and Shimizu, 2005); ageing and the removal of infected, damaged or misplaced cells (Nooden and Penney, 2001; Price et al., 2008). Biochemical and the genetic regulations of PCD in animals (apoptosis) have been well studied (Adrain and Martin, 2001; Bratton and Cohen, 2001; Jacobson et al., 1997) but in plants such studies are still in infancy, despite PCD had a vital role to play in plants for combating environmental stress/es and pathogen attack (Doyle et al., 2010). Plants particularly bamboo, show clear cut phenomenon of PCD, after flowering event, however genetic studies related to this aspect are very scanty. Moreover, the genetic factors and molecular mechanisms behind this phenomenon are largely a mystery to botanists and observers alike.

Bamboo an arborescent and perennial grass (with few exceptions) is considered as semelparous, meaning they reproduce (flowers) only once in their lifetime. On broad basis, three different types of flowering patterns are observed in bamboo (www.inbar.int/Board.asp?BoardID=254). These are (1) annual flowering in which the culm remains healthy after flowering; (2) sporadic flowering includes flowering of only some clumps in an area which subsequently die thereafter and (3) gregarious flowering in which the whole population of bamboo had extensive flowers in a particular time frame and eventually die off. Gregarious flowering, a unique phenomenon is triggered by unfavorable factors like environment, nutrition, climate and their genetical/physiological status (Lin et al., 2010).

Recent studies suggested that factors/genes responsible for PCD are mostly conserved throughout the evolution (Collazo et al., 2006; Green and Reed, 1998; Jacobson et al., 1997; Jan et al., 2008; Kerr et al., 1972; Pennell and lamb, 1997; Peters and chin, 2005; Reape and McCabe, 2010; Vaux and Korsmeyer, 1999). Based on the information available from animal PCD/apoptosis (Adrain and Martin, 2001;

Abbreviations: CLSM, Confocal Laser Scanning Microscope; DEPC, Diethylpyrocarbonate; DIG, Digoxigenin; PCD, Programmed cell death; EST, Expressed sequence tag; SSH, Suppressive subtractive hybridization.

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Bratton and Cohen, 2001; Jacobson et al., 1997), we have investigated the mechanism of PCD in plants. Studies on PCD could also open up opportunities for improving biotic and abiotic stress tolerance in plants (Greenberg, 1997; Hofius et al., 2007; Jones, 2001) also may provide new avenues for manipulating cell death in both plant and animal systems (Collazo et al., 2006; Doyle et al., 2010; Green and Reed, 1998; Jacobson et al., 1997; Jan et al., 2008; Kerr et al., 1972; Pennell and Lamb, 1997; Peters and chin, 2005; Reape and McCabe, 2010; Vaux and Korsmeyer, 1999).

In the present study using the gregarious flowering event of *B. arundinacea* (flowering cycle 44–49 years which lasts for almost a year), attempts were made to identify gene/s (ESTs) accountable for the programmed cell death in bamboo. We used suppressive subtractive cDNA hybridization (SSH) technique, of which cDNA obtained from control *B. arundinacea* internode (5th) was used as driver and that from the test samples (45 days after setting of seeds) as tester. Eleven (11) *in-silico* predicted PCD-related ESTs were analyzed among control and test samples of *B. arundinacea* to determine their differential expression patterns. *In-situ* studies were performed to validate the differential expression patterns of two selected genes namely, V2Ba48 and V2Ba19 between control and test samples. Time-course experiments were performed using the same set of genes to check their expression-kinetics in the test samples collected within a window of 30 to 100 days after setting of seeds. Further, RNA slot-blot was performed using V2Ba48, V2Ba19, V2Ba95 and V2Ba89 ESTs to determine their differential distribution patterns among control and test samples of several other independent plant species namely, *Oryza sativa, Arabidopsis thaliana* and *Nicotiana tabacum*. Taken together, this study may be considered as an initial but systematic step to identify gene/s having either direct or indirect role in programmed cell death-linked en-mass death of bamboo.

2. Materials and methods

2.1. Plant samples

Tissues from 5th internodes of control and test samples of *B. arundinacea*, at different time frames after setting of seeds (Supplementary Fig. 1), grown in natural tropical condition (soil containing 12.5% sand, 50% clit, 37.5% clay, 336.25 kg/ha Nitrogen, 396.48 kg/ha Potassium; 28.17 kg/ha Phosphorous; 1.19% organic carbon and pH 4.9) were collected from eastern region of India (latitude 20° 11′ N and longitude 85° 40′ E). *A. thaliana*, *N. tabacum* and *O. sativa* plants were grown under greenhouse conditions (photoperiod: 16/8 h at 220 μmole m^{-2} s^{-1}; temperature: 28º ± 3 °C. Humidity: 70–75%). Both control (healthy) and test internodal tissues of *N. tabacum* (21 days after setting of seeds), *O. sativa* (21 days after setting of seeds) and *A. thaliana* (14 days after setting of seeds) were collected respectively.

3. Methods

3.1. Isolation of RNA

Total RNA was isolated using the internodal tissue (5th) of control and test samples of *B. arundinacea* respectively using a protocol reported by Rai et al. (2010), treated with *DNase*I (Cat# M6101, Promega, Madison, WI, USA) and purified subsequently. The DNA-free RNA was quantified spectrophotometrically (NanoPhotometer, Sl. No. 1137, Implen, UK). The A_{260}/A_{280} absorption ratio of RNA obtained from control and test internodal tissue ranged between 1.78 and 1.93. The quality of RNA was analyzed in denaturing agarose gel (Sambrook et al., 1989).

3.2. Subtraction cDNA library construction

The mRNA fractions from control and test samples were purified from total RNA using PolyATract® mRNA Isolation System III kit (Cat# Z5300, Promega, USA) following the supplied protocol. Subsequently, each sample was processed for the first strand cDNA synthesis; followed by *Rsa*I-digestion, subtractive hybridization and eventually PCR amplification using Suppressive Subtraction Hybridization kit (Cat# 637401, Clontech, Palo Alto, CA, USA) following manufacturer's instruction. The forward suppressive subtraction library was prepared using cDNA obtained from the internodal tissues of test samples of *B. arundinacea* as tester and that from control internodes as driver. PCR products were purified using Qiaquick PCR purification kit (Cat# 28004, Qiagen, Tokyo, Japan), ethanol precipitated, cloned into pGEMT Easy-Vector (Cat# A1360, Promega, Madison, WI, USA) and transformed into TB-1 *E. coli* cells. Positive clones were identified through colony PCR using SP6 (Q5011, Promega, Madison, WI, USA) and T7 (Q5021, Promega, Madison, WI, USA) primers and sequenced (Sanger et al., 1977). The passed sequences were analyzed using NCBI BLASTX (www.ncbi.nlm.nih.gov/BLAST/) non-redundant protein sequence database (nr) for their categorization as functional proteins (known/ predicted) and proteins with no function (non-significant). The ESTs with known protein functions were further categorized into different protein families using Pfam 24.0 software (www.sanger.ac.uk/resources/software/). These ESTs were further annoted using Blast2go annotation tool (http://www.blast2go.org/).

3.3. Molecular analysis of mRNA transcripts

a) *RNA slot-blot hybridization*

Individual probes were prepared for 11 selected ESTs (Table 1, marked as *) and *β-actin* using primers (Supplementary Table 1) following the protocol described by Rai et al. (2011). An aliquot of 5.0 μg of the total RNA isolated from control and test internodal samples of *B. arundinacea* respectively was blotted on to nylon membrane (Lot# 06 G02009, IMMOBILON NY^+, Millipore, Billerca, MA, USA) and hybridized with ^{32}P-dATP labeled probe at 42 °C overnight and subsequently processed following the protocol of Sambrook et al. (1989) and Rai et al. (2011).

b) *qRT-PCR*

First strand cDNA was prepared by First Strand cDNA Synthesis kit (Cat# A1260, Promega, Madison, WI, USA), using *DNase*I treated RNA obtained from control and test samples of *B. arundinacea*. Each qRT-PCR reaction mixture was prepared using 1 : 20 diluted (approx 100 ng) first strand cDNA and 1 μM gene-specific primer (Supplementary Table 1) in a final volume of 20 μl as per the instructions in QuantiFast SYBR Green RT-PCR kit (Cat# RT-SY2X-03 + WOUN, Eurogentec, Seraing, Belgium). The PCR reactions were carried out using the protocol described earlier (Rai et al., 2011). To handle the limitations of SYBR green technology melting curve analysis was performed at higher temperature (80–85 °C) to nullify the possibility of primer–dimer formation. The threshold cycle (C_t) value of original amplicons were determined for both control and test samples using respective primers: test (Supplementary Table 1) and *β-actin* (Supplementary Table 1) in each case were evaluated. The ΔC_t in both cases (control and test) was obtained by subtracting the C_t value of *β-actin* amplicon from that obtained for test primer, the $\Delta\Delta C_t$ was obtained by subtracting ΔC_t value of the control from test sample and the differences in the transcript level (in folds) between the control and test samples were calculated using $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Moreover, we checked the stability of reference (*β-actin*) gene individually in control and test samples by electrophoresis of respective PCR product on 2% agarose gel. The fold difference in the transcript levels of each gene was presented as the mean of three independent experiments with respective standard deviation (SD).

Table 1
Functional annotation of some important putative PCD specific ESTs obtained from forward SSH library of *Bambusa arundinacea* and their respective roles in programmed cell death based on literature search. Clone name, their respective GenBank Accession numbers and size (in bp) are also listed.

Sl. no.	Clone no.	Accession no.	Functional annotation	E-value	Size (in bp)	Role in programmed cell death
1	V2Ba4*	JG450181	Nucleophosmin, isoform CRA_b	9.00E-35	335	NPM1 is a crucial gene to consider in the context of the genetics and biology of cancer. NPM1 is frequently overexpressed, mutated, rearranged and deleted in human cancer.
2	V2Ba59	JG450182	Nucleophosmin 1 isoform 1	9.00E-35	335	
3	V2Ba6	JG450183	Similar to nucleophosmin 1 isoform 2	9.00E-35	335	
4	V2Ba95*	JG450210	Ubiquitin carboxyl-terminal esterase L1, isoform CRA_c	8.00E-78	521	Ubiquitin activating enzyme/ Cullin controls non-lysosomal mediated protein degradation and thus cell death
5	V2Ba87*	JG450211	Cullin 2	9.00E-119	624	
6	V2Ba83	JG450217	Cullin-4A	5.00E-124	741	
7	V2Ba23	JG450184	Cullin 4A isoform 1	5.00E-121	696	
8	V2Ba74	JG450186	Tu translation elongation factor, mitochondrial, isoform CRA_b	4.00E-82	787	Activated during Cancer
9	V2Ba19*	JG450187	glycogen phosphorylase	1.00E-56	795	Not reported
10	V2Ba31	JG450188	NADH dehydrogenase subunit 4	1.00E-82	686	It is an enzyme located in the inner mitochondrialmembrane and is a potent source of reactive oxygen species.
11	V2Ba85	JG450191	ATP synthase F0 subunit 6	2.00E-39	736	
12	V2Ba39	JG450189	unnamed protein product (putative K + ion channel)	1.5e	390	Controls PCD via formation of permeability transition pore between inner and outer membrane of mitochondria
13	V2Ba42	JG450190	DnaJ (Hsp40) homolog, subfamily B, member 4, isoform CRA_b	2.00E-27	200	
14	V2Ba69*	JG450197	Transcription factor Dp-1	1.10E-01	618	DP1 function as binding partners for E2F transcription factors. Active E2F1/DP1 promotes apoptosis in both a p53-dependent and independent manner.
15	V2Ba26	JG450206	Signal transducer and activator of transcription3 interacting protein	2.00E-59	347	
16	V2Ba76	JG450207	Signal transducer and activator of transcription3 interacting protein 1, isoform CRA_b	3.00E-64	360	Controls lysosomal-mediated cell death *in vivo*.
17	V2Ba89*	JG450208	Nebulin isoform 2	2.00E-109	621	Not reported
18	V2Ba47	JG450209	Serine/threonine-protein kinase SRPK3	2.00E-04	562	
19	V2Ba58	JG450215	Putative serine protease	1.00E-69	625	Short term activation leads to secretion and ion-influx.
20	V2Ba55*	JG450192	Serine arginine rich protein-specific kinase 3 isoform 2	2.00E-04	562	Sustained activation induces apoptosis.
21	V2Ba72	JG450194	Protein kinase C theta	1.00E-137	687	
22	V2Ba48*	JG450214	Aldehyde dehydrogenase 2 family (mitochondrial)	2.00E-23	165	ADH2 mainly plays a role in oxidation of aldehydic substrates but can also function as esterase and reductase and thus may lead to the production of nitric oxide (NO)
23	V2Ba93	JG450216	Rhamnose biosynthetic enzyme 1, putative, expressed	2.00E-123	801	
24	V2Ba112*	JG450220	Peroxisomal D3,D2-enoyl-CoA isomerase	3.00E-89	627	Not reported
25	V2Ba67*	JG450196	Methyl-coenzyme M reductase alpha subunit		408	
26	V2Ba109*	JG450218	TGFB1-induced anti-apoptotic factor 1	2e-49	699	

c) *Semi quantitative RT-PCR*

First strand cDNAs (as discussed earlier) from both control and test samples of *B. arundinacea* were used as individual templates for PCR amplifications of 11 ESTs (as mentioned earlier) using gene specific primers (Supplementary Table 1) following earlier protocol (Rai et al., 2011). The *β-actin* gene was also amplified corresponding to each gene specific RT-PCR reactions using specific primer pair (Supplementary Table 1) as control.

d) *In-situ hybridization*

In-situ hybridization was carried out following earlier protocol Braissant and Wahli (1998) and Corput et al. (1998) with slight modifications. Thin cross sections of control and test internodal samples of *B. arundinacea* were fixed in the FAA (50% ethanol + 10% formalin + 5% acetic acid) solution for an hour. Digoxigenin (DIG)-labeled sense (control) and antisense RNA probes of putative PCD-specific genes namely V2Ba19 and V2Ba48 were synthesized using the DIG RNA Labeling Kit (Sp6/T7) (Cat #11175025910, Roche, Mannheim, Germany) according to the manufacturer's protocol. The pre-fixed tissue sections were then hybridized with riboprobes of V2Ba19 and V2Ba48 respectively at 37 °C overnight. Sections were then washed with DIG Wash and Block Buffer Set (Cat #11585762001, Roche, Mannheim, Germany) as per manufacturer's instruction. The hybridized DIG-labeled mRNA was detected by incubating the sections with anti-digoxigenin antibody conjugated rhodamine (Cat #11207750910, Roche, Mannheim, Germany) diluted by 1: 4 times for 1 h at ambient temperature in the dark (to avoid photo-bleaching or quenching). After washing, the fluorescence of

rhodamine was analyzed under CLSM, using protocol described by Rai et al. (2011).

3.4. Differential expression kinetics of V2Ba19 and V2BA48 in test samples obtained at different time points

Individual probes were prepared for V2Ba19, V2Ba48 and *β-actin* genes using specific primer pairs (Supplementary Table 1) following the protocol described by Rai et al. (2011). An aliquot of 5.0 μg of the total RNA isolated from control and different test samples (30 days, 45 days, 60 days and 100 days) of *B. arundinacea* were blotted on to nylon membrane (Lot# 06 G02009, IMMOBILON NY$^+$, Millipore, Billerca, MA, USA), hybridized at 42 °C overnight in the presence of respective probes and finally processed (Rai et al., 2011). Further, the membranes were de-probed using 1% SDS at 90 °C for 5 min and re-probed with *β-actin* probe to ensure equal loading.

3.5. RNA slot-blot analysis of V2Ba19, V2Ba48, V2Ba89 and V2Ba95 in other independent plant systems

Total RNA were isolated from control and test tissue from different independent plants (*O. sativa, N. tabacum* and *A. thaliana* respectively) as discussed earlier, quantified and blotted on to nylon membrane (Lot# 06 G02009, IMMOBILON NY$^+$, Millipore, Billerca, MA, USA). Individual ^{32}P-dATP labeled probes were prepared for the four selected ESTs namely V2Ba19, V2Ba48, V2Ba95 and V2Ba89 as described earlier. Subsequently, RNA slot-blot analyses were performed for these four probes individually as described earlier.

4. Results and discussion

Following suppressive subtractive hybridization as discussed in Methods, a total of 231 PCR positive ESTs were generated and analyzed. We observed that ESTs obtained varied in sizes ranging from 150 to 900 bp with a maximum 40% density at around 400 bp. Functional annotations of these ESTs were performed using Blast2go annotation tool (Fig. 1). It was observed that these genes fall under different functional groups as response to stimuli, signaling, death, cell proliferation indicating involvement of different metabolic pathways in regulating plant PCD. Furthermore, 143 ESTs were clustered into 19 contigs and 53 singletons using BIOEDIT tool. Putative functions of the *in-silico* translated protein products of ESTs were assigned using BLASTX non-redundant protein sequence database (nr). BLASTX results showed that 82% gene/EST sequences were non-significant (unidentified proteins) which is perhaps due to the fact that the study related to plant PCD is still in its initial stages. Out of the pool of 231 ESTs, 40 important PCD specific genes (ESTs) were submitted to GenBank (GenBank ID**: JG450181 to 450220**). The protein-types involved in bamboo PCD (Supplementary Table 2) were identified using Pfam 24.0 software (http://pfam.sanger.ac.uk/). The information obtained implies that a wide-range of protein family as heat shock protein, kinase, signal transducers, transporter interact among themselves in a complicated network during the progress of plant PCD. Based on the present study and literature search/s a hand full number of important ESTs having direct/indirect participation in PCD were listed along with their functions (Table 1).

Approximately 12.5% (5 out of 40) of the functional proteins indentified in our study including V2Ba48, V2Ba74 (Tu translation elongation factor), V2Ba31 (NADH dehydrogenase subunit 4) and V2Ba85 (ATP synthase F0 subunit 6) were found to be of mitochondrial origin and thus assured the distinct role/involvement of mitochondria in plant PCD. The same was reported earlier; where mitochondria serves as a sensor of death signal (Scott and Logan, 2008), an initiator of the biochemical processes (Dzyubinskaya et al., 2006; Logan, 2008; Logan et al., 2007) and as a genetic regulator of PCD (Desagher and Martinou, 2000; Green and Reed, 1998; Yao et al., 2004).

Further, we observed differential expression patterns of few PCD-related genes namely; V2Ba55, V2Ba95, V2Ba89, V2Ba48, V2Ba87, V2Ba109, V2Ba112, V2Ba4, V2Ba19, V2Ba67 and V2Ba69 (of which the majority were well studied as PCD-related genes: Supplementary Table 3) among control and test samples of *B. arundinacea* (Fig. 2 and Supplementary Fig. 2). Based on the expression levels of 11 ESTs/genes (Supplementary Tables 2 and 3) we categorized them into four major groups: highly expressing (V2Ba48: Aldehyde dehydrogenase2; V2Ba109: TGFB1 induced anti-apoptotic factor 1; V2Ba112: Peroxisomal D-2, D-3 enoyl CoA isomerase; V2Ba19: Glycogen phosphorylase), moderately expressing (V2Ba95: Ubiquitin thioesterase; V2Ba89: Nebulin isoform 2 and V2Ba87: Cullin 2), least expressing (V2Ba67: Methyl CoM reductase alpha and V2Ba69: DP1 transcription

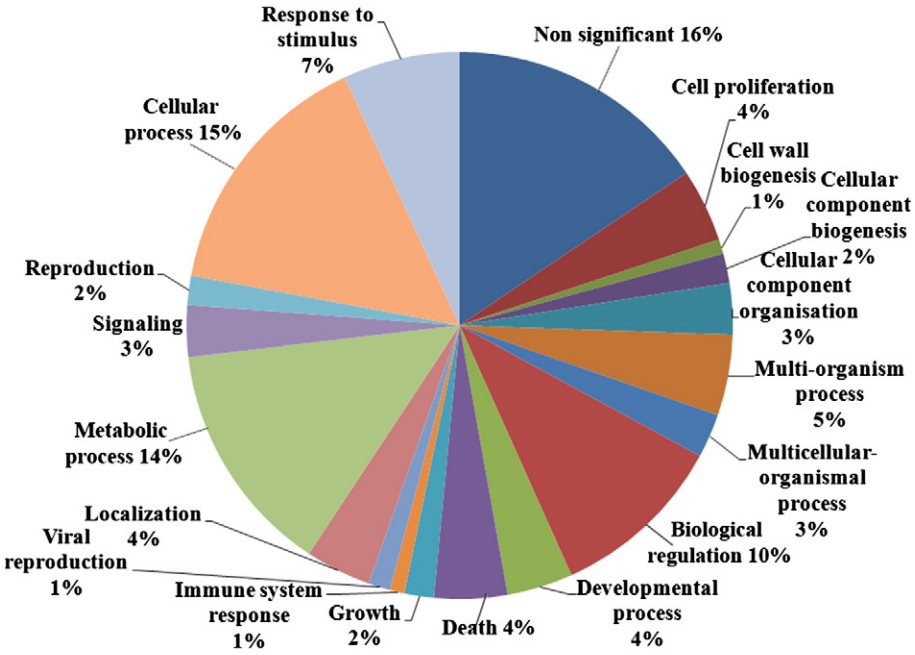

Fig. 1. Functional categorization of putative PCD-specific ESTs of *Bambusa arundinacea* annotated using Blast2go annotation tool (http://www.blast2go.org/).

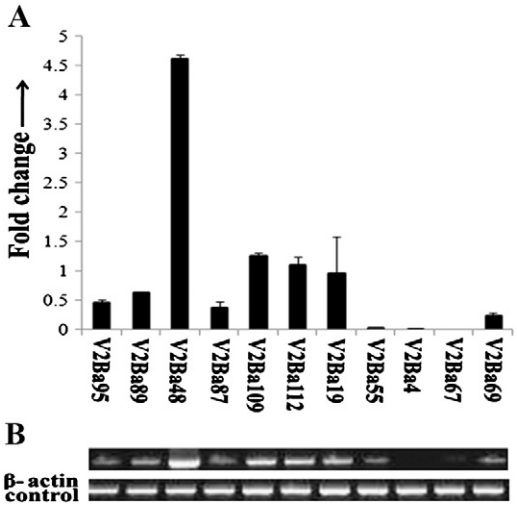

Fig. 2. Relative expression patterns of 11 putative PCD-specific genes (Table 1, marked *) of *Bambusa arundinacea* in control and test internodal samples using qRT-PCR (A) and semi-quantitative RT-PCR (B).

factor, substrate of ubiquitin protein ligase) and that with basal level of expression (V2Ba55: serine arginine rich protein-specific kinase 3 isoform 2). These findings manifest the involvement of delicate regulatory/metabolic pathways involving several genes in an orchestral manner during the process of programmed cell death in plants.

The elevated expression level of V2Ba95 (Ubiquitin thioesterase, the ubiquitin activating enzyme), V2Ba87 (Cullin 2, a ubiquitin protein ligase) and V2Ba69 (Transcription factor DP1, substrate of ubiquitin protein ligase) in test samples as compared to control signifies the involvement of non-lysosomal protein degradation pathways in plant PCD, similar to that observed in case of animal system (Rock and Goldberg, 1999). The up-regulation of V2Ba55 (Serine arginine rich protein-specific kinase 3 isoform 2), key players in the

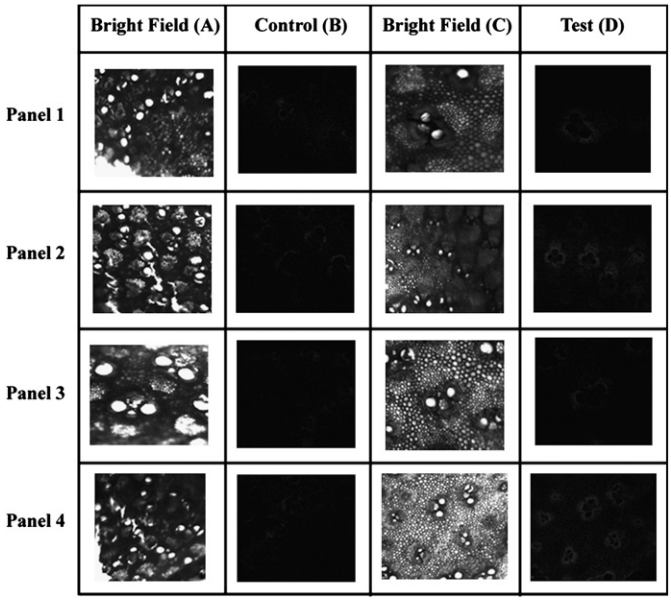

Fig. 3. A CLSM based study to detect *in-situ* localization of a putative PCD-specific gene V2Ba48 (Aldehyde dehydrogenase 2) and V2Ba19 (Glycogen phosphorylase) between control and test internodal samples of *Bambusa arundinacea*, as described in methods. The control images obtained by hybridization with sense V2Ba48 and V2Ba19 RNA DIG-11-UTP labeled probes are presented. Bright field images of each sample are also presented.Both test and control samples were treated with sense V2Ba48 sense probe (Panel 1); V2Ba48 anti-sense probe (Panel 2); V2Ba19 sense probe (Panel 3) and V2Ba19 anti-sense probe (Panel 4) respectively.

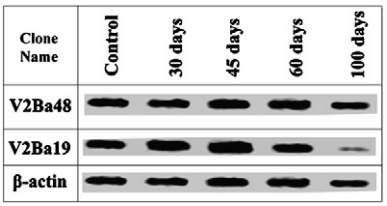

Fig. 4. Detection of the differential levels of expression of bamboo PCD-specific genes namely V2Ba48 (Aldehyde dehydrogenase 2 family) and V2Ba19 (Glycogen Phosphorylase) among control and test samples of *Bambusa arundinacea* collected at different time points; 30 days, 45 days, 60 days and 100 days respectively. *β-actin* gene was used as control.

regulation of alternate splicing (Duque, 2011) suggests that the bamboo plant was under stress condition during PCD.

Results of *in-situ* hybridization showed elevated expression levels of both V2Ba19 and V2Ba48 in the test samples, which were in fine tune with earlier observations obtained from corresponding transcript analysis (Fig. 2). We observed weak intensities of signal in case of control samples using antisense (Fig. 3, Panels 2B and 4B) and sense-RNA (Fig. 3, Panels 1B and 3B) probes. Intensities were found to be almost equivalent in all control samples. Also, a very weak signal was obtained when the control samples were probed with antisense-RNA of V2Ba48 and V2Ba19 respectively (Fig. 3, Panels 2B and 4B). In contrast, stronger signals were obtained in test samples (Fig. 3, Panels 2D and 4D) when probed with antisense RNA of V2Ba48 and V2Ba19. Suggesting, elevated level of expression of these genes during bamboo PCD.

Expression studies of V2Ba19 (glycogen phosphoryase) and V2Ba48 (aldehyde dehydrogenase 2) at different time points (30, 45, 60 and 100 days after setting of seed) showed consistent expression levels of V2Ba48 (Fig. 4). This perhaps is due to the fact that one of its (V2Ba48) functions is to act as esterase and reductase (Chen et al., 2010; Guillen and Evans, 1994; Hofmann et al., 1999; Liu and Schnable, 2002) leading to the production of nitric oxide (NO), an essential signaling molecule (Van Breusegem and Dat, 2006) required throughout the process of PCD. While, V2Ba19 demonstrated differential expression level during bamboo cell death; at 45 days time point it showed maximum expression while its least expression was detected at 100 days, suggesting involvement of glycogen phosphorylase during the early phases of bamboo PCD.

Differential expression levels obtained for four genes namely V2Ba19, V2Ba48, V2Ba89 and V2Ba95 using different plant species namely *O. sativa, N. tabacum* and *A. thaliana* clearly indicated that V2Ba95 was highly expressed in the test samples of *O. sativa* and *N. tabacum* while a relatively lower expression was seen in the test samples of *A. thaliana* (Fig. 5). Similarly, V2Ba48 was expressed in all the samples studied with maximum expression in the test samples of *O. sativa* followed by *N. tabacum* and *A. thaliana* while the expression of V2Ba19 and V2Ba89 was indistinguishable in all the cases studied (Fig. 5). These observations indicate that these genes are involved in

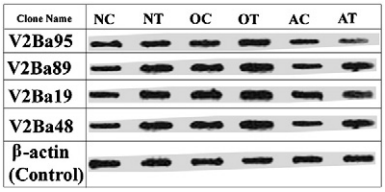

Fig. 5. Detection of differential transcript levels of bamboo PCD-specific genes namely V2Ba95 (Ubiquitin thioesterase), V2Ba89 (Nebulin isoform 2), V2Ba48 (Aldehyde dehydrogenase 2 family) and V2Ba19 (Glycogen Phosphorylase) among control and test samples of *Nicotiana tabacum, Oryza sativa* and *Arabidopsis thaliana* using RNA slot-blot. NC: *Nicotiana tabacum* Control; NT: *Nicotiana tabacum* Test; OC: *Oryza sativa* Control; OT: *Oryza sativa* Test; AC: *Arabidopsis thaliana* Control; AT: *Arabidopsis thaliana* Test (details about the collection of samples in Materials and methods section) *β-actin* gene was used as control.

PCD like cell death in both monocot and dicot plants suggesting their global distribution.

Surprisingly, in the present study we did not identify any homologs of flowering-related genes/ESTs as seen in other studies (Abe et al., 2005; Hisamoto and Kobayashi, 2007; Lin et al., 2010). This may be the case as the test samples were collected 45 days after setting of seed i.e. the bamboo sample might have crossed the flowering phase and have entered into the early PCD phase.

5. Conclusion

Taken together, from molecular perspective the phenomenon of bamboo cell death after setting of seed is a transcriptionally and physiologically controlled process, associated with differentially expressed genes/genetic factors and proteins, working in a delicate genetic network. Present study has identified few PCD-related/specific genes of fundamental importance which could play a vital role in the elucidation of underlying mechanism involving programmed cell death.

Supplementary materials related to this article can be found online at doi:10.1016/j.gene.2012.01.018.

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