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Cloning of differential expression fragments in cauliflower after *Xanthomonas campestris* inoculation

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Abstract

A near isogenic line (NIL) of *Brassica oleracea* var. *botrytis* with resistant and susceptible lines C712 and C731, was used in this study. More than 100 differentially expressed cDNA fragments were obtained from black rot resistant cauliflower plants obtained using cDNA-amplified fragment length polymorphism (AFLP) after infection with the pathogen. Thirteen of these fragments were cloned and subjected to reverse Northern blot analysis using both infected and control cDNA pools. Two positive clones, M2 and M6, were isolated. Northern dot blot and Northern blot analyses showed that M2 was constitutively expressed, whereas M6 contained a gene that was differentially expressed during pathogen infection. Moreover, M6 cDNA fragment was also highly expressed 16 - 24 h after H_2O_2 treatment. Southern blots showed that M6 is a single copy gene in the cauliflower genome, and encodes a protein with 84 % homology to gene on *Arabidopsis* chromosome 1. The deduced M6 protein has 91 % positive homology with the *Arabidopsis* 2A6 protein, which regulates ethylene synthesis; 76 % homology with a 1-aminocyclopropane-1-carboxylate oxidase (ACO), the last enzyme in ethylene synthesis; and 70 % homology with an ethylene induced DNA binding factor. These results suggest that M6 gene fragment is a new H_2O_2 downstream defense related gene fragment and can be induced by *Xanthomonas campestris* pv. *campestris* and H_2O_2.

Additional key words: black rot resistance, *Brassica oleracea* var. *botrytis*, cDNA-AFLP, H_2O_2 treatment.

Introduction

Xanthomonas campestris is a gram-negative, pathogenic bacterium that belongs to the γ-subdivision of Proteobacteria. It is genetically differentiated into over 141 pathovars (pv.), each with a specific host range. The variant *X. campestris* pv. *campestris* (*Xcc*) generally invades and multiplies in cruciferous plant vascular tissues, including broccoli, cabbage and cauliflower, resulting in the characteristic "black rot" symptoms of blackened veins and V-shaped necrotic lesions at the foliar margin (Alvarez 2000). Periodic epidemics of black rot disease have occurred worldwide, especially in the developing regions of Africa and Asia, where high temperature and humidity can aggravate the damage (Stall 1993). Each epidemic can cause substantial yield loss in agricultural production.

Plants are confronted with a variety of natural pathogens (virus, fungi and bacteria). The success of a pathogen in infecting a host plant depends on how rapidly the plant recognizes the pathogen and activates appropriate defence reactions. If the pathogen carries an *avr* (avirulence) gene whose product is specifically recognized by the product of the corresponding *R* (resistance) gene in the plant, resistance mechanisms are triggered rapidly, resulting in disease resistance. If either the *avr* or the *R* gene is absent, the pathogen is not recognized rapidly, the defence responses are activated slowly, and disease ensues (Dangl and Jones 2001). Recognition of a potential pathogen often results in a hypersensitive reaction (HR) and the activation of programmed cell death (PCD) of cells at the site of the attack, designed to stop the spread of the pathogen (Glazebrook 2001).

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Abbreviations: ACO - 1-aminocyclopropane-1-carboxylate oxidase; CTAB - hexadecyltrimethylammonium; cDNA-AFLP - cDNA-amplified fragment length polymorphism; DIG - digoxygenin; ET - ethylene; HR - hypersensitive reaction; ISR - induced systemic resistance; JA - jasmonic acid; LD-PCR - long distance PCR; NIL - near isogenic line; PCD - programmed cell death; PCR - polymerase chain reaction; PVP - polyvinylpyrrolidone; SAR - systemic acquired resistance; *Xcc* - *Xanthomonas campestris* pv. *campestris*.
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Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are each involved in the regulation of basal resistance against different pathogens (Repka *et al.* 2004, Dubery *et al*. 2007). These three signalling molecules play important roles in induced resistance as well. SA is a key regulator of pathogen-induced systemic acquired resistance (SAR), whereas JA and ET are required for rhizobacteria-mediated induced systemic resistance (ISR). Both types of induced resistance are effective against a broad spectrum of pathogens (Ton *et al.* 2002).

To date, the model plant *Arabidopsis thaliana* has been used extensively in studies of the processes leading to black rot resistance. In particular, mechanisms of SAR, and the concomitant signal pathway activation and defense-related gene expression, have received a great deal of attention. However, black rot disease resistance is not well characterized in cauliflower *(Brassica oleracea* var. *botrytis)*. Black rot genetics has been examined in the related model plant *Arabidopsis thaliana* by following segregation of resistance to *Xanthomonas campestris* pv. *campestris* (*Xcc*)-in the ecotype Columbia. The data suggested that not only is black rot resistance a monogenic trait, but that its inheritance can be followed via QTL analysis (Jun *et al*. 1991; Buell *et al*. 1997). A number of pathogenesis-related genes have been cloned from this interaction. For example, a class IV chitinase gene (*AtchitIV*) from *Arabidopsis thaliana* has been cloned. This result suggests an involvement of *AtchitIV* in the initial events of the hypersensitive reaction (Liliane *et al.* 1997). *Athsr* gene families have been identificated and have shown to be specifically or preferentially expressed during the HR (Christophe and Dominique 1999). The relationship between SA and ET in basal resistance to *Xcc* has also been examined in *Arabidopsis* (Ton *et al.* 2002). Moreover, some defence-related genes were carried out (Martine *et al.* 2002, Chern *et al.* 2001, François *et al.* 2000).

The aim of this paper was to study gene expression in black rot-resistant cauliflower following mock and *Xcc* inoculation. One cDNA fragment, M6, was found to have an influence on black rot resistance. Further analysis revealed that M6 cDNA fragment expression could be induced by H_2O_2 treatment.

Materials and methods

Plants and treatments: Near isogenic lines (NILs) of *Brassica oleracea* L. var. *botrytis* (cauliflower) were provided for this study by Tianjin Kernel Vegetable Research Institute (Tianjin, China). C712 is resistant to *Xcc* and C731 is susceptible to this pathogen.
The control group is mock-inoculated resistant line by *Xcc*. The treatment group is resistant line using *Xcc* infected past 6 d, which has resistant reaction.

For plant inoculation, pathogen (*Xanthomonas campestris* pv. *campestris*) was grown in PSA medium (peptone 10 g dm^{-3}, sucrose 10 g dm^{-3}, glutamic acid 1 g dm^{-3}) at 28 °C in a shaking (200 rpm) inocubator until the pathogen concentration reached 10^8 cells cm^{-3}.

Four-week-old cauliflower plants were split into two groups, one for control and one for plant inoculation. The treatment group was inoculated by clipping leaf tips with sterile scissors dipped in *Xcc* cultures (10^8 cells cm^{-3}). The control group was mock inoculated by clipping leaf tips with sterile scissors dipped in sterile water. After inoculation, the plants were kept in a greenhouse at 23 - 25 °C, with a relative humidity >95 %.

The same materials were used for H_2O_2 treatment experiment. 3 % H_2O_2 solution and sterile water were sprinkled on leaves of treatment and control group, respectively. Plants were grown as described above.

DNA and RNA isolation: Total DNA was isolated by hexadecyltrimethylammonium (CTAB) according to Murray and Thompson (1980) with some modifications. Fresh leaves (0.2 g) of both lines were ground in liquid nitrogen. The frozen powder was directly added to 2 cm^3 lysis buffers (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 1.4 M NaCl, 0.2 % β-mercaptoethanol, 2 % PVP and 1× CTAB). After incubation at 56 °C for 30 min, 2 cm^3 phenol: chloroform: isoamyl alcohol (25:24:1) was added. The supernatant was obtained by centrifugation at 10 000 *g* for 10 min and extracted once with an equal volume of chloroform:isoamyl alcohol (24:1). After centrifuging at 11 000 *g* for 15 min, equal volume of cold isopropanol was added to the supernatant. The mixture was incubated for 30 min at -20 °C. DNA was pelleted by centrifugation at 11 000 *g* for 30 min at 4 °C and dissolved in 0.1 cm^3 TE (pH 8.0) buffer. RNA was removed from the DNA by treatment with 200 mg cm^{-3} RNase A for 1 h at 37 °C.

Total RNA was isolated by the single-step method using acid guanidinium throcyanate-phenol-chloroform extraction (Chomczynski and Sacchi 1987). Contaminating DNA was digested by DNase I (*TaKaRa*, Otsu, Shiga, Japan) for 30 min at 37 °C. The RNA was checked for quality and quantity by 1.2 % formaldehyde denaturing agarose gel and fluorimetry (*ND-1000*, *NanoDrop*, delaware, USA) and stored at -70 °C.

cDNA synthesis and cDNA-AFLP procedure: The first strand of cDNA was synthesized using *SMART*™ polymerase chain reaction (PCR) cDNA synthesis system (*Clontech*, San Jose, USA) according to the manufacturer's instructions. Full-length double strand cDNA was synthesized by long distance PCR (LD-PCR). LD-PCR was performed in a total volume of 0.05 cm^3 containing 0.001 cm^3 the first strand of cDNA, 0.005 cm^3 10× cDNA PCR buffer (*Clontech*), 0.001 cm^3 dNTP (10 mM each, *Sangon* Shanghai, China), 0.001 cm^3 $T_{23}MN$ (0.5 μM) and SCSP (0.5 μM) primers, respectively, and 0.001 cm^3 50× advantage cDNA polymerase

mix. The primer sequences of T_{23}MN and SCSP are as follows: T_{23}MN primer: 5'-TTTTTTTTTTTTTTTTTTT TTTTMN-3', N (A, T, G or C), M (A, C or G), SCSP primer: 5'-CTCTTAATTAAGTACGCGGG-3'.

PCR amplification reactions were performed in a thermal cycler (*MG5331*, *Eppendorf*, Hamburg, Germany) using the following program: initial denaturation step at 95 °C for 1 min, followed by 25 cycles of 95 °C for 30 s, 65 °C for 30 s and 68 °C for 6 min. PCR products were separated by electrophoresis on a 1 % agarose gel, stained with ethidium bromide (0.8 μg cm^{-3}), and visualized under UV radiation.

The cDNA-amplified fragment length polymorphism (AFLP) method was performed essentially according to Vos *et al* (1995) with minor modifications. Briefly, 100 - 150 ng of cDNA was digested with 1.5 U of both *Eco*R I and *Mse* I (*Sangon*). After ligation and pre-amplification, selective amplification was conducted by combining 30 ng of both *Eco*R I primers and *Mse* I primers containing three selective nucleotides. Thermo-cycling was done with 35 cycles and including a 12 cycle touchdown (annealing temperature was reduced from 65 °C to 56 °C in 0.7 °C increments for 12 cycles and subsequently maintained at 56 °C for 23 cycles). Amplification products were separated on 6 % denaturing polyacrylamide gels running at 30 W for 1.5 h in 1× TBE buffer. After silver staining (Bassam *et al.* 1991), the gels were dried at room temperature and photographed. In this paper, twenty selective primer pairs were used; *Eco*R I and *Mse* I adaptor sequences plus two or three selective nucleotides respectively. The primer combinations are showed in Table 1.

Table 1. Polymorphism analysis of cDNA-AFLP amplification products generated with 20 different primer combinations.

Primer combinations	Number of bands	Number of poly-morphic bands	[%]	Number of mono-morphic bands
E-GA+M-CTT	23	3	13.0	20
E-GA+M-CTC	26	10	38.5	16
E-GA+M-CTA	31	18	58.1	13
E-GA+M-CTG	21	5	23.8	16
E-GA+M-CAA	19	6	31.6	13
E-AC+M-CTC	17	9	52.9	8
E-AC+M-CTG	22	5	22.7	17
E-AC+M-CTA	16	8	50.0	8
E-AC+M-CTT	27	13	48.1	14
E-AC+M-CAA	20	7	35.0	13
E-AG+M-CTT	23	7	30.4	16
E-AG+M-CTC	12	5	41.7	7
E-AG+M-CTG	26	8	30.8	18
E-AG+M-CTA	17	6	35.3	11
E-AG+M-CAA	21	6	28.6	15
E-AA+M-CTT	30	8	26.7	22
E-AA+M-CTG	24	8	33.3	16
E-AA+M-CTC	18	6	33.3	12
E-AA+M-CTA	21	4	19.0	17
E-AA+M-CAA	20	5	25.0	15
Total	434	147	33.9	287

Cloning of cDNA fragments: Fifteen candidate differentially expressed cDNA bands (out of one hundred differentially expressed bands) were excised from denaturing polyacrylamide gels, eluted in 0.03 cm^3 of sterile water and boiled for 5 min. Thereafter, the supernatant was obtained by centrifugation at 12 000 *g* for 10 min. 0.005 cm^3 of the supernatant was used as template in 0.05 cm^3 reaction volume with the PCR primers used in the selective amplification. 40 cycles were as follows: 94 °C 30 s, 57 °C 30 s and 72 °C 1 min. PCR products were separated on a 1 % agarose gel, and thirteen DNA fragments of the appropriate size were re-amplified. The target bands were purified by the *QIAEXII* gel extraction kit (*Qiagen*, Valencia, CA, USA), ligated to the pUCm-T easy vector (*BBI*, Kitchener, Canada) and then transformed into competent cells of *Escherichia coli* DH5α by heat shock (Sambrook *et al*. 1982, Evers *et al*. 2006). Positive clones were identified by PCR methods.

Reverse Northern blot analysis: Reverse Northern blots were performed as described by Vögeli *et al*. (1996, 1997) and Li *et al*. (1998) with some modifications. Clones containing candidate cDNA fragments were dotted onto nylon membranes (*Pall*, NY, USA). Total cDNA derived from the control and treatment group was labelled with digoxygenin (DIG)-11-dUTP using the random primer DNA labelling system (*Roche*, Grenzach, Germany), Hybridization of probes to membranes and detection of signal was performed following the instructions of the *Roche* DIG high prime DNA labelling and detection starter kit II. The membrane was hybridized at 42 °C overnight and was washed at high-stringency. Twice in 2× SSC/0.5 % SDS solution at 42 °C for 30 min, followed by two further twice washes in 0.5× SSC/0.5 % SDS solution at 65 °C for 15 min.

Northern dot blot: To further check the quantitative sequence expression of M2 and M6 clones, Northern dot blot was executed. The 25 μg denaturing total RNA derived from control and treatment samples, which infected after 6 d, were dotted onto a nylon membrane (*Pall*), respectively. Then hybridization was conducted with M2 and M6 cDNA clones, which was labelled with [α-^{32}P] dCTP using PCR labelling method. The wash procedures for the Northern dot blots were carried out at 42 °C. The blots were washed twice with a 2× SSC/0.1 % SDS solution for 5 min then twice with a 0.2× SSC/0.1 % SDS solution for 15 min. X-ray film was exposed to the filter at -20 °C (Radwan *et al*. 2004).

Southern and Northern blot: Southern blot analysis was used to determine the copy number of M6. M6 was labeled with DIG-11-dUTP. Cauliflower genomic DNA that was digested with *Sau*3A I or *Eco*R I (*Sangon*), respectively. Genomic DNA fragments separated on 0.8 % agarose gel and then transferred to the nylon

membrane (*Pall*). The blots were probed with DIG-11-dUTP labeled M6 as described previously. Southern blots were washed at 50 °C. The membrane was washed twice with a 2× SSC/0.1 % SDS solution for 10 min and then with a 0.5× SSC/0.1 % SDS solution for 20 min. Hybridization signals were detected following the manufacturer's instructions of random DIG prime DNA labelling and detection kit (*Roche*).

Total RNA, extracted from control and treatment group, was separated under denaturing conditions on 1.2 % agarose gel containing formaldehyde, and then transferred to a nylon membrane (*Pall*), in accordance with standard techniques (Sambrook *et al*. 1982). The M6 clone was labelled with [α-^{32}P] dCTP using PCR labelling method. After hybridization, the nylon membrane was washed under conditions of high stringency. The wash procedures for the northern blots were carried out at 42 °C. The blots were washed twice with a 2× SSC/0.1 % SDS solution for 5 min then twice with a 0.2× SSC/0.1 % SDS solution for 15 min. X-ray film was exposed to the filter at -20 °C (Radwan *et al.* 2004).

Sequencing and sequence analysis: M6 clone was sequenced at *Sangon* using the *PRISM* ready reaction *DyeDeoxy* termination cycle sequencing kit and an *ABI-3700* DNA sequencer (*Applied Biosystems*, Foster City, USA). Nucleotide and deduced amino acid sequences derived from M6 was compared to *GenBank* deposits using *BLAST* (Altschul *et al*. 1997).

Results

cDNA-AFLP analysis of cauliflower infected by *Xcc*: Total RNA was isolated from plant infected with *Xcc* for 4 or 6 d and without *Xcc* treatment (Fig. 1). Differential expression in these three samples was analyzed using cDNA-AFLP. Total 434 clearly and highly resolution bands were obtained from twenty primer combinations including 147 polymorphism markers. The number of polymorphic markers per primer pair ranged from 10 to 30. Of the total number of markers generated, 33.9 % were polymorphic markers.

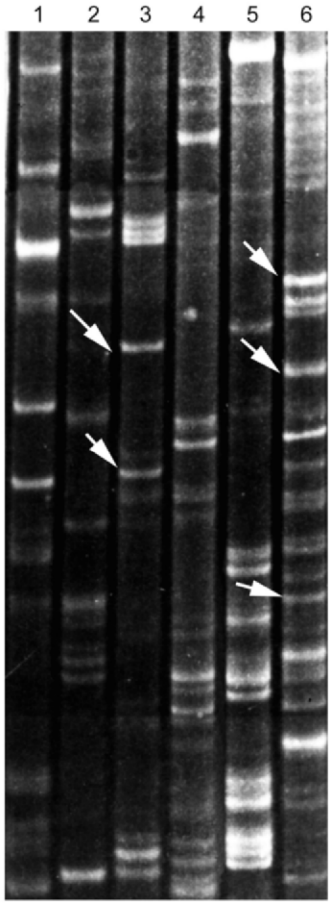

Fig. 1. Resistant line expression pattern at differential inoculation stages (4 and 6 d) using two primer pairs. *Lane 1* and *4* - control group, *lane 2* and *5* - treated group 4 d after inoculation, *lane 3* and *6* - treated group 6 d after inoculation. The *arrows* indicate several differential bands.

These polymorphic markers can be divided into three groups: *1*) their presence is induced by pathogen, *2*) they are absent after infection, and *3*) they are constitutively expressed during pathogen infection, but expression level is different.

Reverse Northern blot analysis: cDNA confirmation by reverse Northern blot analysis was carried out on thirteen positive clones (derived from fifteen differentially expressed bands) by hybridization with control and treatment groups. Three different sets of results were observed. The first set contained M1, M3, and M4 clones. Hybridization signals indicated that these cDNA fragments are constitutively expressed (data not shown). The second contains M5 and M7 - M13 clones for no signal was detected (data not shown). These eight clones were believed to be either false positive or they have very low copy numbers. Thus, they should be tested further using Northern blots. The third set includes the M2 and M6 clones. The M2 clone exhibited a strong signal with the treatment group and a weak signal with the control group, while the M6 clone hybridization signal was only seen with the treatment group (data not shown). These results confirmed that the M2 and M6 clones contained differentially expressed sequences.

Expression of M2 and M6 cDNA fragments: Northern dot blot analysis was used and the M6 clone hybridized only to the treatment group (data not shown). However, the M2 clone produced strong signals with both treatment and control groups. These results indicate that M2 was constitutively expressed during pathogen infection, whereas M6 was truly differentially expressed.

M6 cDNA fragment copy number: To determine the copy number of the M6 DNA sequence in the cauliflower genome, Southern blot analysis using the resistant line was performed. Genomic DNA was digested with *Sau*3A I and *Eco*R I and hybridized with a DIG-11-dUTP labelled probe generated from a M6 cDNA fragment. Only one band was observed for each of the endonucleases tested. These results suggest that the M6 cDNA fragment belongs to a single copy gene (Fig. 2).

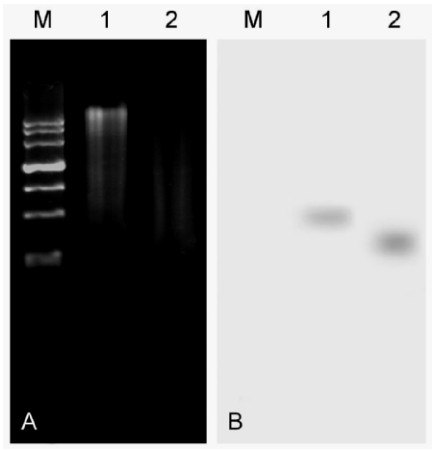

Fig. 2. Southern blot hybridization analyses of M6 cDNA fragment within cauliflower genome. *A* - Image of cauliflower genomic restriction enzymes digestion products. *B* - DNA samples from cauliflower genome were digested *Eco*RI and *Sau* 3AI, transferred to nylon membrane and probed with M6 clone. *Lane M* - DNA molecular mass marker, *lane 1* - *Eco*RI-digested cauliflower genomic DNA, *lane 2* - *Sau* 3AI-digested cauliflower genomic DNA.

Expression of M6 cDNA fragment after *Xcc* infection: Expression of the M6 cDNA fragment was tested using Northern blot analysis. The transcript of M6 cDNA fragment was detected among RNA from the treatment group. No signal was seen in the control group (Fig. 3). It should be noted that the M6 transcript is expressed during the resistance process and that this transcription can be induced and controlled.

Functional determination of M6 cDNA fragment: H_2O_2 was used to test whether expression of the M6 cDNA fragment could be induced by a stimulus other than pathogen infection. A physiological reaction was observed in leaves after approximately 24 h of exposure to H_2O_2. Many cell death-like or hypersensitive reaction-like disease spots were found on leaf surfaces after 16 - 18 h of H_2O_2 treatment (data not shown). All of the leaves died rapidly after 24 or 48 h. Total RNA was isolated from leaves 1, 6, 12, 16 and 24 h after H_2O_2 treatment. Northern blots were performed (Fig. 4). Interestingly, the results suggest that M6 transcript accumulated early after H_2O_2 treatment, and a strong accumulation was seen 16 to 24 h after H_2O_2 treatment. No expression was observed between 1 to 12 h. All of these findings suggest that the M6 cDNA fragment is a downstream gene fragment that can be induced by pathogen or signalling molecule.

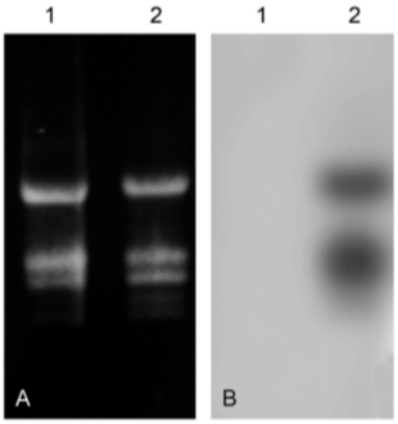

Fig. 3. Northern blot hybridization analysis of M6 cDNA fragment. *A* - Image total RNA of control and infected by *Xcc* group. *B* - Northern blot hybridization of control and treated group total RNA with [α-^{32}P]dCTP labeled M6 clone. *Lane 1* - control group, *lane 2* - treated group.

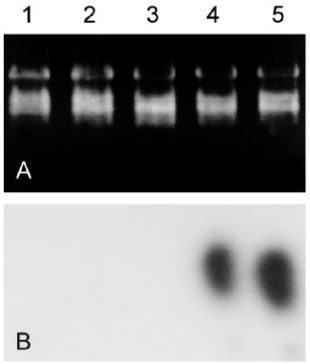

Fig. 4. Effects of H_2O_2 stress on M6 cDNA fragment expression. *A* - A resistant line total RNA image at different H_2O_2 treatment stages. *B* - Northern blot hybridization with [α-^{32}P]dCTP labeled M6 cDNA fragment. *Lane 1* - H_2O_2 treatment 1 h, *lane 2* - H_2O_2 treatment 6 h, *lane 3* - H_2O_2 treatment 12 h, *lane 4* - H_2O_2 treatment 16 h, *lane 5* - H_2O_2 treatment 24 h.

Sequencing and sequence analysis: Sequencing result suggest that M6 cDNA fragment full sequence is 159 bp. Sequence and deduced protein analysis of M6 cDNA fragment performed in *GenBank* found an 84 % homology with part of BAC F19P19 on chromosome 1 of *Arabidopsis thaliana,* which encodes part of the 2A6 protein. The deduced protein structure indicates that M6 cDNA fragment is 91 % homologous with the *Arabidopsis* 2A6 protein, a regulator of ethylene synthesis; 76 % homologous to 1-aminocyclopropane-1-carboxylate oxidase (ACO), which is the last enzyme in ethylene synthesis; and 70 % homologous with an ethylene induced DNA binding factor (Fig. 5).

Fig. 5. Alignment of the translated cauliflower (*Brassica oleracea* var. *botrytis*) M6 cDNA fragment.

Discussion

Arabidopsis 2A6 protein encodes a protein of 361 amino acids. Southern blot analysis indicates that the corresponding gene is unique in the *Arabidopsis* genome (Trentmann *et al*. 1995). Three highly conserved domains of ACO were inspected. It is structurally related to the enzyme that catalyzes the last step in ethylene synthesis and is a regulator of ethylene synthesis (Liu *et al.* 1997). But previous study indicated that ACO is a gene family including ACO_1, ACO_2 and ACO_3 three members. The β-glucuronidase (GUS) reporter gene was put under control of the ACO_1 and ACO_3 promoters to examine their regulation in transgenic tobacco plants. The ACO_1 promoter was rapidly induced (within 8 - 12 h) in tobacco leaves inoculated with the hypersensitive response-inducing bacterium *Ralstonia solanacearum*. This result suggests that ACO_1 can be induced by pathogen infection and that ethylene plays an important role in the signalling pathway (Lasserre *et al*. 1997).

M6 cDNA fragment was cloned in cauliflower and appears to be induced by *Xcc* and H_2O_2. The M6 cDNA fragment was highly expressed during early-induction by H_2O_2 (16 - 24 h). At the same time, programmed cell death was observed at the site of the attack. It has been reported that H_2O_2 is a reactive oxygen intermediate normally generated during the course of a plant-pathogen interaction. H_2O_2 was also identified that is early signal generated during the oxidative burst (Coego *et al.* 2005). In tomato plants, H_2O_2 has been demonstrated to function as a second messenger mediating the systemic expression of various defence-related genes (Orozco-Cardenas *et al.* 2001). The expression of the *OsRac1* mRNA, homology of mammalian Rac-GTPase, in response to signalling components JA and H_2O_2 were examined in wild-type rice seedling. The *OsRac1* transcript, whose accumulation required certain *de novo* synthesized protein factor(s), increased in the leaves upon H_2O_2 treatment, but not by JA (Agrawal 2003). In addition, the oxidative burst in incompatible pathogen-challenged *Arabidopsis* leaves was found to activate a secondary systemic burst in distal parts of the plants, leading to the establishment of systemic immunity via the expression of defence-related genes (Alvarez *et al.* 1998).

In this paper, H_2O_2 treatment and sequence analysis results suggest that M6 gene fragment is H_2O_2 downstream defence related-gene fragment and can be induced by *Xcc* and H_2O_2. M6 gene fragment is a new defence relative gene fragment in cauliflower. Further experiments are required for obtaining M6 gene full sequence and the elucidation of detailed mechanisms.

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