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Cloning of Ethylene Responsive Genes from the Apices of Cucumber Plants (*Cucumis sativus* L.)

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Abstract

The technique of differential display was used to isolate ethylene-responsive cDNAs from the apices of cucumber plants. Differences in RNA populations from apices treated with or without ethephon (an ethylene-releasing compound) were examined using 80 primer combinations. Northern blot analysis confirmed that 20 cDNAs represented mRNAs that were differentially expressed upon ethephon treatment (promoted, 17; suppressed, 3). Sequence analysis of these cDNAs revealed that two clones were identical to the 3'-terminal regions of the *CR20* and *CUS3* genes of cucumber. Five clones showed significant similarity to the C-terminal regions of short-chain alcohol dehydrogenases, β-1,3-glucanases, *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, products of the *Aux/IAA* gene family, and lipoxygenases. The other 13 clones were found to have no significant homology in the databases. Because ethylene promotes female flower formation in cucumber plants, some of 20 cDNAs might be involved in processes of sex regulation.

The phytohormone ethylene orchestrates a variety of physiological processes in plants, including senescence, fruit ripening and abscission (Abeles *et al.*, 1992; Lelièvre *et al.*, 1997). It also plays an important role in physiological responses to environmental stresses such as water deficit, mechanical wounding and pathogen attack (Abeles *et al.*, 1992). One of the interesting effects of ethylene is in relation to sex regulation in cucumber plants (*Cucumis sativus* L.). Treatment of cucumber plants with ethephon, an ethylene releasing compound, or with gaseous ethylene, promoted the development of female flowers (McMurray and Miller, 1968; Rudich *et al.*, 1969; Iwahori *et al.*, 1970). Furthermore, a high correlation was found between the evolution of ethylene from apices and the formation of female flowers (Rudich *et al.*, 1972; 1976). Previous studies have revealed a number of ethylene-regulated genes *via* differential screening or differential display techniques. Whilst these have aided in understanding the molecular mechanisms of ethylene action and in defining the role of this hormone in physiological processes (reviewed in Deikman, 1997; Zegzouti *et al.*, 1999), they have not addressed the role of ethylene in the regulation of sex expression in plants. Therefore, we attempted to identify novel genes regulated by ethylene during female flower-bud induction in the apices of cucumber plants, using the differential display technique. In this study, we describe the isolation of 20 partial cDNA clones that correspond to ethylene responsive genes, as determined by their change in abundance during the induction of female flower formation, following treatment with ethephon.

We selected a monoecious cultivar (*Cucumis sativus* L., cv. Shimoshirazu-jibai) for the plant material. Genetically, Shimoshirazu-jibai plants have a stable male tendency on the lower nodes of the main stem. The cucumber plants were grown in soil-filled pots in a phytotron at 25℃ during 14 h of light and at 20 ℃ during 10 h of dark. In order to induce female flower formation, the apices of 15-day-old-Shimoshirazu-jibai seedlings (two- to three-leaf stage) were treated with or without 500 μM ethephon in a 0.02% (v/v) Tween 20 solution. A piece of absorbent cotton, soaked in the appropriate solution, was applied to the apex of each cucumber plant, and this treatment was repeated on three consecutive days. After growth of the seedlings, the sex of each flower on the first 17 nodes was then examined and classified as male or female. A node was designated male if it had at least one male flower and it was designated female if only female flowers were present. As shown in **Table 1**, Shimoshirazu-jibai plants showed stable maleness at the lower nodes of the main stem, and no female

Table 1 Effect of ethephon treatment on sex expression in monoecious cucumber plants (cv. Shimoshirazu-jibai).

Treatment*	Number of nodes per plant		
	Female	Male	Aborted
Control	0.0 ± 0.0	15.9 ± 0.1	1.1 ± 0.1
Ethephon	8.0 ± 0.5	7.8 ± 0.5	1.3 ± 0.3

The sex of each flower on the first 17 nodes of the main stem was examined and classified as male or female. Results for 8 plants (± SE) are given. *The apices of 15-day-old seedlings (cv. Shimoshirazu-jibai) were treated with or without 500 μM ethephon solution for 3 days.

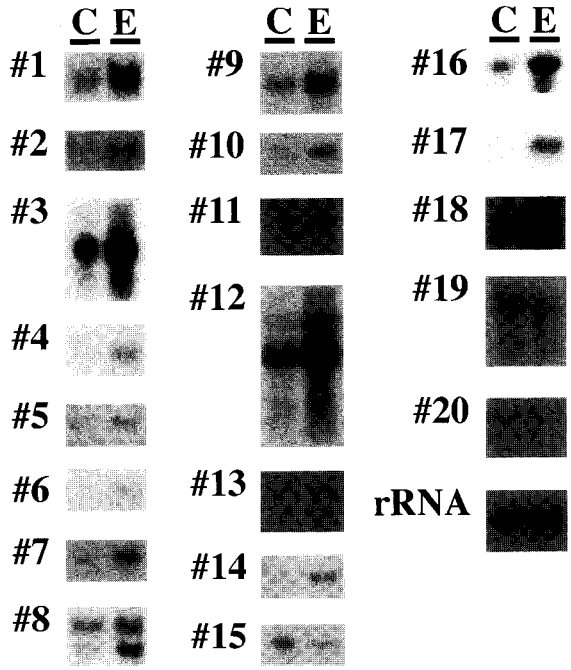

Fig. 1 Northern blot analysis of 20 cDNAs obtained by differential display.
The apices of 15-day-old seedlings (cv. Shimoshirazu-jibai) were treated with (E) or without (C) 500 μM ethephon for 3 days. The apices were collected at 18 days after sowing. Total RNA (20 μg) was extracted from each apex, separated on a denaturing agarose gel, transferred to a nylon membrane, and allowed to hybridize with each cDNA probe. The blot was re-hybridized to an rRNA probe as a gel-loading control. C; control, E; ethephon treated

flowers were formed at the lower nodes in the control experiment. When the apices of Shimoshirazu-jibai plants were treated with ethephon, female flowers were induced on the nodes of the main stem (**Table 1**). In order to obtain samples for differential display, the apices of 15-day-old seedlings that had been treated with or without ethephon for three days were excised from the seedlings, just above the youngest leaf, and frozen immediately in liquid nitrogen. Total RNA was extracted from the apices of cucumber plants as described by Prescott and Martin (1987), and then the RNA was purified by successive precipitations from lithium chloride. Prior to use for differential display, the extracted total RNA was purified by cesium chloride density gradient ultracentrifugation at 200,000x g for 16 h. The purified RNA was then treated with DNase (RQ1 RNase-Free DNase, Promega Inc., Madison, WI, U.S.A.) to exclude DNA contamination. The differential display screening procedure was performed as described by Liang *et al.* (1993). Eighty sets of differential display reactions were performed using 20 arbitrary decamer combinations with four anchor primers ($T_{12}MA$, $T_{12}MT$, $T_{12}MG$, and $T_{12}MC$, where M stands for a mixture of G, A, and C) according to the manufacturer's protocol (RNAmap™, mRNA differential display system, GenHunter Co., Brookline, MA, U.S.A.). The cDNA was synthesized in a 60-μl reaction mixture containing 0.6 μg of total RNA, 20 μM dNTPs, 150 units of StrataScript™ Reverse Transcriptase (StrataGene, La Jolla, CA, U.S.A.) and 1 μM of one of the anchor primers at 37 ℃ for 50 min. The template and the primer were denatured at 65 ℃ for 5 min prior to reaction. The reaction was stopped by heating at 95 ℃ for 5 min. One-tenth of the reverse-transcription mixture was used as a template in a PCR reaction containing a corresponding $T_{12}MN$ primer (1 μM) in combination with one of the 20 arbitrary 10-base primers (0.2 μM) for each reaction. PCR was performed using 1 unit of AmpliTaq® (Perkin-Elmer Japan Co. Ltd., Urayasu, Japan) in a 20-μl reaction mixture containing [α-^{32}P]dCTP (ICN Biomedicals Inc., Costa Mesa, CA, U.S.A.) and 2 μM dNTPs. The parameters for PCR were 40 cycles of denaturation at 94 ℃ for 30 sec, annealing at 40 ℃ for 2 min, and extension at 72 ℃ for 30 sec, followed by a final extension at 72 ℃ for 5 min, using a GeneAmp® PCR system 9600 (Perkin-Elmer Japan Co. Ltd.). Aliquots of duplicate reaction mixtures after PCR were subjected to electrophoresis on a 6% polyacrylamide/8 M urea sequencing gel to separate the amplified cDNAs. This resulted in the identification of 92 differentially expressed cDNA bands (62 promoted and 30 suppressed) detected in a series of experiments. The regions of the gel containing the differentially expressed cDNAs were excised from the dried gel, and each was eluted with 100 μl of distilled water. The eluted cDNAs were then reamplified with the appropriate pair of primers. Reamplified PCR prod-

Table 2. Characteristics and sequence analysis of cDNAs isolated by differential display.

cDNA number	Length (bp)	Ethylene regulation	Transcript size (kb)	Accession number	Sequence similarity*	
						Accession number****
#1	170	Induction	1.2	AB051366	short-chain alcohol dehydrogenase, putative [*Arabidopsis thaliana*] (S=40, E=0.004)**	AC037424
#2	334	Induction	2.5	AB051367	None found	
#3	209	Induction	1.3	AB051368	None found	
#4	362	Induction	2.5	AB051369	None found	
#5	311	Induction	1.5	AB051370	None found	
#6	335	Induction	2.4	AB051371	None found	
#7	360	Induction	1.2	AB051372	basic extracellular β-1,3-glucanase precursor [*Vitis vinifera*] (S=100, E=6e-21)**	AF053750
#8	540	Induction	2.8, 1.7	AB051373	*CR20* gene for noncoding RNA [*Cucumis sativus*] (S=922, E=0.0)***	D79217
#9	208	Induction	2.0	AB051374	None found	
#10	265	Induction	1.5	AB051375	None found	
#11	206	Induction	2.2	AB051376	None found	
#12	206	Induction	2.1	AB051377	None found	
#13	102	Induction	2.6, 1.6	AB051378	None found	
#14	101	Induction	1.5	AB051379	None found	
#15	138	Suppression	1.4	AB051380	None found	
#16	386	Induction	1.4	AB051381	*S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase [*Clarkia breweri*] (S=52, E=2e-06)**	AF133053
#17	456	Induction	1.0	AB051382	*CUS3* gene for putative transcription factor [*Cucumis sativus*] (S=872, E=0.0)***	AJ278013
#18	402	Induction	1.1	AB051383	None found	
#19	375	Suppression	1.4	AB051384	*Nt-iaa4.1* deduced protein [*Nicotiana tabacum*] (S=100, E=1e-20)**	AF123509
#20	213	Suppression	2.3	AB051385	probable lipoxygenase, CPRD46 [*Vigna unguiculata*] (S=55, E=1e-07)**	T11578

The nucleotide sequences reported in this paper, #1 to #20, have been submitted to DDBJ under accession number AB051366 to AB051385, respectively. *BLAST scores (S) and 'expect' value (E) were obtained during a similarity search in the GenBank database. ** Determined using the BLASTX program. *** Determined using the BLASTN program. **** GenBank accession number of the homologous gene.

ucts were cloned into a pCR™II vector by the method described in the TA Cloning® Instruction Manual (Invitrogen, San Diego, CA, U.S.A.).

RNA gel blot analysis, using the same samples of RNA as used for the differential display, was performed to confirm that the genes corresponding to the screened clones are differentially expressed. To prepare cDNA probes, the cDNAs that were cloned by differential display were excised from the pCR™II vector with *Eco*RI, and purified by gel electrophoresis. The cDNA fragments were labeled with [α-^{32}P]dCTP using a Multiprime™ DNA labeling system (Amersham Pharmacia Biotech UK Ltd., Bucks., U.K.) prior to use as probes. Total RNA (20 μg per lane) was separated by electrophoresis on a 1.17% agarose gel containing 0.66 M formaldehyde, and was then transferred to a Gene-Screen *Plus*® membrane (NEN Life Science Prod-

ucts Inc., Boston, MA, U.S.A.) by capillary action with 10x SSC (1.5 M sodium chloride/ 0.15 M sodium citrate), as recommended by the manufacturer. The RNA was immobilized on the membrane by baking at 80℃ for 30 min. The blots were hybridized with each cDNA probe, as described by Kamachi *et al.* (1997). Ribosomal RNA was used as an internal standard to ensure that equal amounts of total RNA were present in each lane. Autoradiographs of the blots revealed that many clones represented either false-positive or undetectable RNAs. However, twenty clones (#1 - #20) showed differences in the accumulation of their transcripts in the apices treated with ethephon (**Fig. 1**). The expression of transcripts corresponding to clones #15, #19 and #20 was down regulated, whilst the remaining 17 genes were up regulated by ethephon treatment. The cDNAs were sequenced with a DNA sequencer (model 377; Perkin-Elmer Japan Co. Ltd.) using the dideoxy sequencing method (Sanger *et al.*, 1977) and a Taq Dye Primer Cycle Sequencing kit (Perkin-Elmer Japan Co. Ltd.). Nucleotide and deduced amino acid sequences were analyzed with GENETYX-MAC software, version 10.1.1 (Software Development Co., Tokyo, Japan). Databases were searched using NCBI's BLAST (National Center for Biotechnology Information, Basic Local Alignment Search Tool version 2.0). The search revealed that 13 clones did not correspond to known genes in the database (**Table 2**). The lack of homology to known sequences for most of the clones could be because they were only partial-length cDNAs. These cDNAs theoretically correspond to the 3' ends of mRNA, which also contain non-coding sequences of variable length, and thus there may be insufficient sequence length of these clones for a meaningful search. On the other hand, the other 7 clones showed significant similarity with several known genes (**Table 2**). Clones #8 and #17 showed nearly 100 % identity at the nucleotide level to the cucumber genes *CR20* and *CUS3*, respectively, throughout the sequences that we had isolated. Thus, these two clones are expected to be partial sequences representing each corresponding gene. A noncoding RNA is transcribed from the *CR20* gene, which is repressed by cytokinin treatment of cucumber cotyledons, although its function is not understood well (Teramoto *et al.*, 1996). This is the first report to show that the accumulation of *CR20* transcripts is stimulated by ethylene. The *CUS3* gene has only been recorded as a partial sequence in the database, and was isolated from embryogenic callus. *CUS3* showed similarity with some MADS-box genes and was considered to encode a putative transcription factor. Therefore, it is likely that clone #17 encodes a member of the

Fig. 2 Alignments of deduced amino acid sequence of #1 (A), #7 (B), #16 (C), #19 (D) and #20 (E).

The clones that scored 100 and below in **Table 2** were aligned with each similar gene. Amino acid is indicated as a single letter code. Asterisks indicate terminal codon. Pluses and dots indicate identical and similar residues. The clone names and accession numbers of similar genes are shown at left side. AC037424; putative short-chain alcohol dehydrogenase (*Arabidopsis thaliana*), AF053750; basic extracellular β-1,3-glucanase precursor (*Vitis vinifera*), AF133053; *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (*Clarkia breweri*), AF123509; *Nt-iaa4.1* deduced protein (*Nicotiana tabacum*), T11578; probable lipoxygenase CPRD46 protein (*Vigna unguiculata*)

MADS-box gene family. For the remaining clones (#1, #7, #16, #19 and #20), their deduced amino acid sequences showed significant similarity to the C-terminal regions of short-chain alcohol dehydrogenases, β-1,3-glucanases, *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, *Nt-iaa4.1* deduced protein (a member of *Aux/IAA* gene family) and lipoxygenase, respectively (**Table 2** and **Fig. 2**).

In this study, we reported that 20 cDNA clones showed ethylene-responsive expression in apices of cucumber plants. Each of the 20 clones may be involved in different physiological events that are influenced by ethylene. For example, β-1,3-glucanase is known to be a PR-2 (pathogenesis-related-2) protein, and its mRNA accumulation is induced by ethylene (reviewed in Bol *et al.*, 1990; Vögeli *et al.*, 1988). Thus, clone #7 may be involved in the plant's defense mechanism against fungal infection. However, some genes that act in a cascade during sex expression of cucumber plants may be included in the 20 cDNAs too. The family of short-chain alcohol dehydrogenase genes includes the *TASSELSEED2* gene, which may be involved in sex determination in maize (DeLong *et al.*, 1993). Since several MADS-box genes regulate reproductive organ development (reviewed in Angenent and Colombo 1996), clones #1 and #17 are likely candidates for genes that are associated with the sex expression of cucumber plants. Isolation of full-length sequences of their cDNAs and a more detailed study of their expression would be required before further speculation can be made. However, the information that we have obtained in this study should prove useful for further studies of the mechanism of the action of ethylene during female flower induction in cucumber plants.

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