

# キュウリ果実の水ストレス誘導ポリガラクトナーゼ遺伝子の 発現解析

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## Expression of a Water Stress-induced Polygalacturonase Gene in Harvested Cucumber Fruit

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### Summary

We investigated the molecular response of harvested cucumber (*Cucumis sativus* L.) fruit to postharvest water stress. Using RT-PCR, we isolated *CUPGI* cDNA, a cDNA clone encoding polygalacturonase (PG) that is inducible by water stress in cucumber fruit and characterized the expression of the gene with respect to hormonal control. The full length sequence determined by RACE-PCR, encoded a deduced protein of 435 amino acids that contained several common conserved domains as reported for PGs. The *CUPGI* gene expression was also induced by exogenous ethylene but not by the application of abscisic acid (ABA). The level of *CUPGI* mRNA induced by water stress, was reduced by 50% in fruits pre-treated with 1-methylcyclopropene (MCP), a strong inhibitor of ethylene action, which suggests that the expression of *CUPGI* gene could be induced by a direct mechanism through water stress and an indirect mechanism mediated through ethylene produced in response to water stress. We conclude that water loss in harvested cucumber fruit results not only in physical changes but also leads to molecular responses, including PG gene expression.

**Key Words:** ABA, ethylene, gene expression, polygalacturonase (EC 3. 2. 1. 15), water stress.

### Introduction

Water loss from harvested fruits and vegetables is one of the critical factors that affect their postharvest quality and shelf life because most crops have no means to replace the water lost through transpiration. A loss in weight of only 5 per cent, in general, will cause many perishable commodities to appear wilted and shriveled. Research on water loss in harvested crops has mainly been focused on physical aspects, such as how water loss occurs and how much loss leads to unacceptable appearance, while physiological effects of water stress have received little attention (Ben-Yehoshua, 1987). Our previous research has demonstrated that rapid water loss induces a low but significant evolution of ethylene in harvested cucumber fruit. This results in flesh softening and a concurrent increase in water soluble pectin and a decrease in hexametaphosphate soluble

pectin (Xue et al., 1996a; 1996b; 1996c). A similar observation was reported in harvested bell peppers (Lurie et al., 1986; Ben-Yehoshua et al., 1983). These results suggest that tissue softening caused by water loss not only involves a reduction of turgor pressure in the cell but also in physiological changes in cell wall components. The modification of pectin in plant tissues has been attributed to the action of PG that catalyzes the hydrolytic cleavage of  $\alpha$ -(1- $\rightarrow$ 4) galacturonan linkage (Fischer and Bennett, 1991). Several cDNAs encoding PG have so far been cloned and characterized in some plant tissues, suggesting that PG genes consist of a multi-gene family (Hadfield and Bennett, 1998). Recent advances in molecular biology technology such as the use of PCR, have enabled us to isolate a target gene and characterize its expression. In this study we used the molecular biological approach in an attempt to understand the textural changes associated with water loss in harvested cucumber fruit. Here we report the induction and expression of a PG gene in response to water stress and a possible involvement of hormonal regulation.

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## Materials and Methods

### Plant material and treatments

Freshly harvested greenhouse grown cucumber (*Cucumis sativus* L. cv. Sharp one) fruits at commercial maturity were obtained from a grower in Kurashiki, Japan. The fruits were sorted by defects and maturity; those of uniform maturity, shape, and size were pretreated with or without MCP, a strong inhibitor of ethylene action (Sisler and Serek, 1997; Serek et al., 1995; Nakatsuka et al., 1997; 1998) and held at 40 % RH and 25 °C. Two sets of fruit were covered with wet tissue paper and plastic film to minimize water loss through transpiration and incubated with or without 1000 ppm ethylene. After a 24 hr incubation, fruits were weighed to calculate their water loss; fruit samples were frozen in liquid nitrogen and stored at -80 °C until analyzed.

To determine the effect of ABA on the expression of the PG gene, fruit segments (10 cm long) were cut from the central part of the fruit and soaked in 1000 ppm ABA or distilled water as described in Mathooko et al. (1998). The segments were vacuum-infiltrated at 400 mmHg for 1 min to facilitate absorption of the solution by the fruit tissues. After the 24 hr incubation at 25 °C, the segments were sampled as above.

### Cloning and sequencing of PG gene

Total RNA was extracted by the hot-borate method according to Wan and Wilkins (1994) with minor modification. Poly (A)<sup>+</sup> RNAs used for RT-PCR and northern analysis were isolated by affinity chromatography using, Oligotex-dT30 (Takara, Kyoto) according to the manufacturer's instructions. The cDNA for RT-PCR was synthesized by AMV reverse transcriptase (Life Tech Co.Ltd. Tokyo), using Poly (A)<sup>+</sup> RNA extracted from water-stressed cucumber fruit as a template. PCR was conducted under the following conditions, 94 °C for 1 min, 40 °C for 1 min, 72 °C for 2 min, for 30 cycles using degenerate primers based on regions of high homology between aligned plant PG cDNA sequences registered in databases. The sequence of the upstream primer was 5'-T(ACGT)(AGT)(ACG)(AT)GG(CGT)CC(AT)TG(CT)A-3' and that of the downstream primer was 5'-AT(ATG)CC(AG)TG(AGT)CC(AT)GG(ATG)CC(AG)CA-3'. The PCR product was cloned into pUC118 vector / *Sma* I and sequenced using an automated DNA sequencer (model DSQ-1000L, Shimadzu, Kyoto). The nucleotide sequence of the full length cDNA was obtained by RACE-PCR method using Marathon cDNA Kit (Clontech, USA) according to the manufacturer's instructions. The deduced amino acid sequence of *CUPG1* (accession no.AB035890) was aligned to 20 full length deduced amino acid sequences of PG homologues using Genetyx-Mac software (Software Development Co. Ltd., Tokyo).

### Southern and northern analyses

Genomic DNA was extracted from young cucumber leaves using the method of Murray and Thompson (1980). Five-microgram of DNA digested with *Eco*RI, *Hind*III or *Xba*I restriction enzymes were fractionated on 0.8% agarose gels and transferred to Hybond-N membrane (Amersham, USA). The resulting blot was hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled probe synthesized using the RT-PCR clone as template. The hybridization was carried out in 50% formamide, 6 x SSPE [1 x SSPE is 0.15 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub> and 1 mM EDTA (pH 7.4)], 5 x Denhard's reagent (1 x Denhard's is 0.02 % each of Ficoll-400, PVP, and bovine serum albumin), 0.5% SDS, and 100 mg·ml<sup>-1</sup> denatured herring sperm DNA at 37 °C. The membrane was washed in 0.2 x SSPE, 0.1% SDS at 55 °C.

The accumulation of mRNA for PG gene was estimated by northern analysis with the radio-labeled probe synthesized in the same manner as for the Southern hybridization, except that hybridization and washing were carried at 42 °C and 60 °C, respectively. Equal mRNA loading was confirmed by rehybridization with a 0.4 kb fragment of actin gene cloned by RT-PCR (Mathooko et al., 1999; accession number, AB010922).

### Effect of MCP-pretreatment on stimulation of ACO activity by propylene

To verify the effectiveness of MCP as a strong inhibitor of ethylene action, its anti-ethylene activity was assayed by *in vivo* 1-aminocyclopropane-1-carboxylate oxidase (ACO) activity in cucumber fruit. MCP-pretreated and non-treated fruits were exposed to 5,000 ppm propylene at 25 °C for 24 hr. Then, transverse slices weighing 2 g were excised from the center part of fruit and *in vivo* ACO activity was determined by measuring the capability of the tissue to convert administered 1-aminocyclopropane-1-carboxylate (ACC) to ethylene as previously described (Mathooko et al., 1993).

## Results and Discussion

We previously demonstrated that postharvest water loss in cucumber fruit caused it to be shriveled and softened, mainly due to reduced turgor pressure (Xue et al., 1996b). We also found that the softening was accompanied by an increase in water-soluble pectin, a decrease in hexametaphosphate-soluble pectin and induction of PG activity (Xue et al., 1996a; 1996b). In harvested bell pepper fruit, Ben-Yehoshua et al. (1983) found that sealing them with plastic film extended their postharvest life of the fruit by inhibiting fruit softening as well as changes in cell wall pectin, and delays disintegration of cellular membranes determined by leakage of amino acids. The beneficial effect of sealing is, however, prevented by including hygroscopic CaCl<sub>2</sub>, suggesting that the sealing effect can not be related to

'modified atmosphere' mechanism but to reduced water stress. Lurie et al. (1986) reported that water-soluble pectin in bell pepper fruit stored in 97% RH remained at the same level as at harvest while in 85% RH it increased twofolds. Taken together, our observations and those of other researchers suggest that postharvest water loss in two nonclimacteric fruits is not only involved in physical changes but also in a physiological change that includes modification of pectic substances in the cell wall. It is against this background that we attempted to clone a PG gene which is responsible for textural changes associated with water stress in cucumber fruit.

#### *Cloning of a gene encoding putative PG and its genome structure*

RT-PCR using conserved domains yielded a 0.48 kb fragment. Although more than 50 RT-PCR clones were investigated, they were identical, sharing high homology in both nucleotide and deduced amino acid sequence with previously cloned PG genes. We designated this clone *pCUPG1*. The full length sequence, obtained by the RACE-PCR using the nucleotide sequence of *pCUPG1*, consisted of an open reading frame of 435 amino acids and included four conserved domains of reported PG homologues in the deduced amino acid sequence (Fig. 1). Phylogenetic analysis grouped the PGs into two major clades, A and B (Fig. 2). *CUPG1* belongs to clade A which includes PGs that are expressed in fruit and/or abscission zones. Clade B primarily comprises exo-PGs expressed in pollen or anthers. The genomic Southern analysis (Fig. 3) yielded one band each in the *HindIII* and *EcoRI* digests and two bands in the *XbaI* digest, although there is no *XbaI* recognition site inside the cDNA sequence. It is unclear whether hybridization to another *CUPG1* homologue caused the second band in the *XbaI* to digest. In general, however, these results suggest that *CUPG1* exist, as a single copy gene in cucumber genome (Takahara et al., 1999), which we attribute to the probability that *XbaI* site in intron is much higher than is the probability that *HindIII* and *EcoRI* digestion yield the same size fragments from two different genes. To verify this possibility, the genomic gene cloning and structural analysis might be the subject of our next study: however, in this paper we proceed discussion on the assumption that *CUPG1* is transcribed from a single copy gene. Hitherto, more than 100 PG homologues have been registered in the databases; PGs appear to form a large multigene family in many plants such as tomato, maize, and melon (Hadfield and Bennett, 1998). Hadfield et al. (1998) isolated six PG homologues from melon and demonstrated that three of them are expressed in the fruit. Surprisingly, all six melon PG genes have less than 50% homology with the *CUPG1* in deduced amino acid sequence although more than 90% homology is found in ACC oxidase and ACC synthase

genes between melon and cucumber which belong to the genus *cucumis* (Shiomi et al., 1998; Shiomi et al., 1999). This mismatch suggests that a subfamily of PG homologues exists within melon and cucumber.

#### *Expression of CUPG1 and its hormonal control*

Many plant genes have been shown to be induced by drought stress and function in drought tolerance (Yamaguchi-Shinozaki et al., 1995); however, PG gene has not been included in the group of genes responsive to desiccation. Most previous studies were targeted to understand the mechanism by which whole on plants survive under a severe environment. To date, studies on postharvest technology in horticultural crops have not been conducted at the molecular level. Northern blot analysis (Fig. 4) show that the accumulation of *CUPG1* mRNA was induced in the fruit, subjected to water stress; the accumulation was reduced to half by a pre-treatment with MCP (Sisler and Serek, 1997; Serek et al., 1995). The gene expression was also detected in the fruit that was incubated in humidified atmosphere in the presence of 1000 ppm ethylene. These expression patterns of *CUPG1* agree well with those of the enzyme activity (Xue et al., 1996b). Increased PG activities have been reported in bell pepper exposed to water stress (Ben-Yehoshua et al., 1983). PG activity and its gene expression have been most extensively studied in ripening tomato fruit to understand pectin hydrolysis in relation to fruit softening. The transgenic tomato fruits, carrying antisense ACC synthase gene, accumulate large amounts of PG mRNA but fail to accumulate the PG polypeptide. This suggests that ethylene controls only the translatability of the PG polypeptide but not the accumulation of its mRNA (Theologis, 1992). Recently, Sitrit and Bennett (1998) concluded that PG mRNA in tomato fruit is ethylene regulated. Our northern analysis indicated that *CUPG1* mRNA is inducible by treating immature cucumber fruit with exogenous ethylene, but when the fruit that was pre-treated with MCP and then exposed to low-humidity atmosphere, only half the level of *CUPG1* mRNA accumulated compared to the untreated control fruit. The assay of ACO activity revealed that 5,000 ppm ethylene (equivalent to 50 ppm ethylene) significantly enhanced the *in vivo* ACO activity (Fig. 5), whereas the pretreatment with MCP completely reversed the action of propylene which suggests that *CUPG1* mRNA accumulation by water stress is, at least in part, dependent on ethylene. Indeed, ethylene production by the cucumber fruit is induced by water stress; but the level is less than  $0.1 \text{ nl} \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$  (Xue et al., 1996c). It is reasonable to postulate that low level ethylene has a limited physiological effect.

The accumulation of ABA has been well-known as a primary response to drought in plants; it contributes to drought tolerance by controlling guard cells (Mansfield, 1987). In this experiment, we found that ABA application had little effect on the *CUPG1* mRNA

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CUPG1      1'          MDKLFLLSLLGLFIIAYGVSGAFVSDSD
MEPG3      1" MCRRLLGRRPCCLSLLTFLFVVVVNFDTFSTCFGSYPVDNPLPSISSGD
CUPG1     29' IDMIKELESFDIEEEDDDVYSYTPVWRSEKILMNVDSDAIGDGV
           ..***...*...***
MEPG3     51" KEDYQGHYEHSSSEFSSMLRTRLEKMVSSPLASPEIFNVDDYGAMGDG-
CUPG1     79' TDDTMAFRKAWETACSTSKSVLLVPKEKTYLVNATTFKGPCKDGMVIQIE
           .*** **...*...***.....*** ...* .....**...***... ..***
MEPG3    100" EDDTEAFKETWKDACSSTNAIFLPCDRVYHLKPITFSGPCNSPLLFQIE
CUPG1    129' GTIVAPDEPELWDPKFPQWLEFTKLNAATFQGDGVIDGSGEKWWAESCK
           **.*.....*...**.*...*...***...**...***...**...
MEPG3    150" GTVKASPHISDYE-KDRRHWIIFQNLLGLRVEGKGIINGNRKWWLNSCK
CUPG1    179' KNKSRPCKGAPTALITDSSSNIRVKGLTIQNSQQMHTIARSETVRITGV
           **.*...***...*...*...**...*...*...*...*...*...*...*...
MEPG3    199" VNKTLPCKEAPTAVTFYQCTNLRVEGLRFRNAQMLLSFQRCNNVKALNL
CUPG1    229' RVSAPGDSSPNTDGIHLTQSTNVVVQNCKISTGDDCISIVNASSGIKMKGI
           ..***.*****.*...*...**.*...*****...*...***
MEPG3    249" WIYAPGNSSPNTDGIHVTGTQFIVIKNCLIMTGDDCISIVSGSKNVRAKGI
CUPG1    279' SCGPGHGISIGSLGKDNSTGIVTKVVLDTAYLRETTNGVRIKTWQGGSGY
           .*****.....*...*...**.*...*...*****...**
MEPG3    299" TCGPGHGISIGSLGAGKSEAEVSNVVVDATAKFSGTSNGVRIKTWQGGKGY
CUPG1    329' VRAVRFENVRMEDVENPIIIDQFYCDSPTTCEQTSAVKISQIMYRNISG
           ....*.*...*****.*...**...*...*...*...*...*...*...
MEPG3    349" AQNIIIFQNIIVMDNVTNPIIINQNYCDQKEPCTQQADAVAVSNVMYQNIRG
CUPG1    379' TTTSKNAMKFACSDSVPCSNIIILSNVLEKTDGTVETYCHAAQGFGLWCC
           *...*...*...**.*...*...*...*...*...*...*...
MEPG3    399" TSASEVAVKFDCKSVPCQGILLQDINLVHKGKNDKSAQAEASCKNVKW
CUPG1    429' APIGETA
MEPG3    449" KNRGRVSPQCGGG

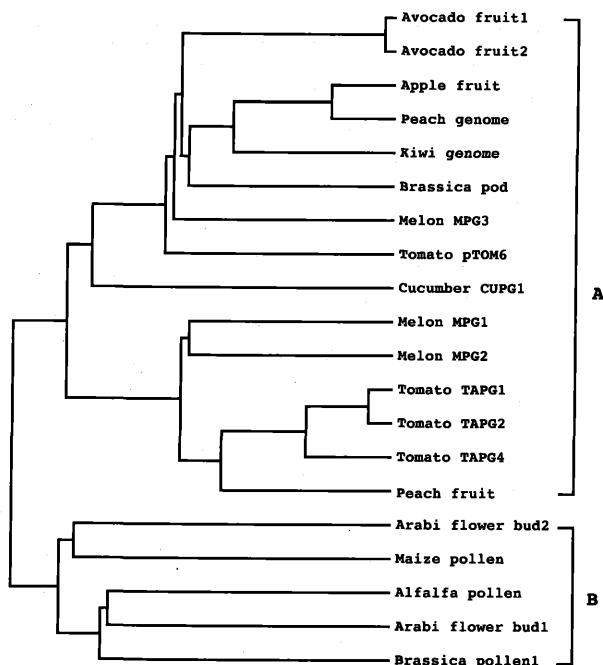
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**Fig. 1.** Sequence analysis of the *CUPG1* and *MEPG3* (melon PG, GeneBank accession, AF062467) and alignment of their deduced amino acid sequences. Asterisks and dots indicate identical and conserved amino acid residues, respectively, between the *CUPG1* and *MEPG3*. The highly conserved domains in PG genes are boxed.

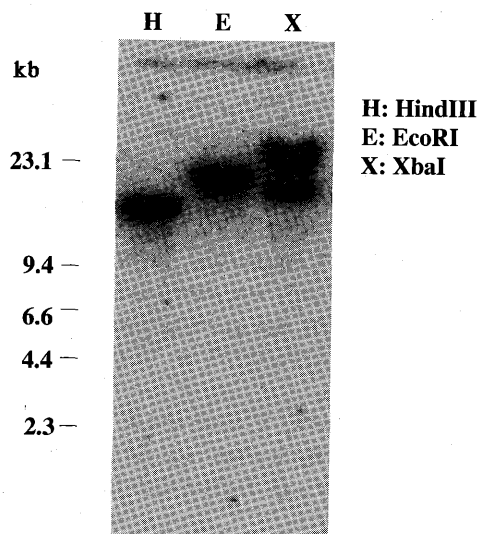
accumulation (Fig. 4). Shinozaki and Shinozaki (1991), their review of plant responses to drought in gene transcription in *Arabidopsis thaliana*, and noted that some drought-responsive genes are induced by ABA, but others are not. In their study of three independent signal transduction pathways, they suggested that two are ABA-dependent, and one is independent. The latter seems to be mediated through second messengers, such

as calcium and protein kinase. The accumulation of *CUPG1* mRNA might respond to water stress through such a signal transduction pathway.

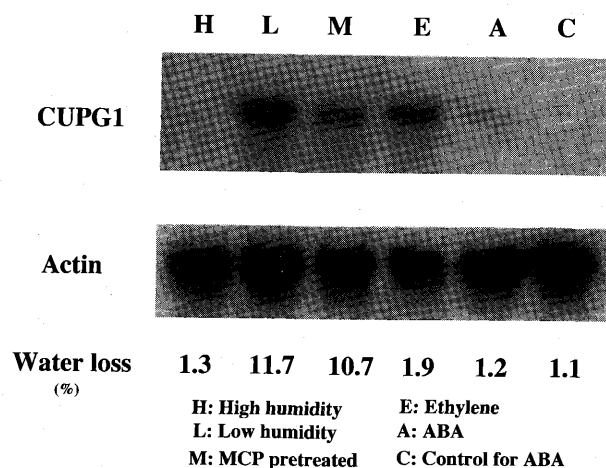
In conclusion, our results suggest that water loss in harvested cucumber fruit results not only in physical changes but also leads to molecular responses, including PG gene expression.



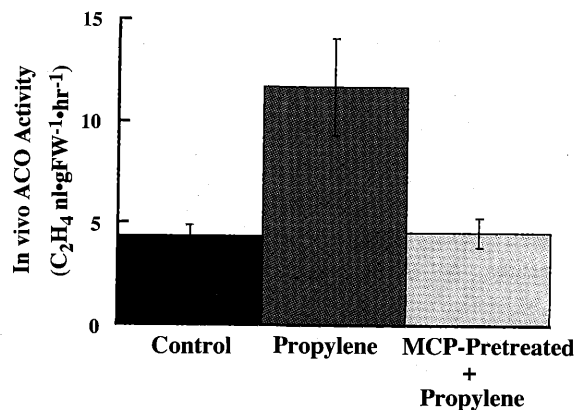
**Fig. 2.** Phylogenetic tree for 20 plant PG cDNAs and genomic clones. The tree was generated from the alignment of the full-length deduced amino acid sequences using Genetyx-Mac software. GeneBank accession number: avocado fruit 1 (L06094); avocado fruit 2 (X66426); apple fruit (L27743); Peach genome (X77231); kiwi genome (L12019); brassica pod (X95800); melon *MEPG3*(AF062467); tomato *pTOM6* (A24194); melon *MEPG1*(AF062465); melon *MEPG2*(AF062466); tomato *TAPG1* (U23053), tomato *TAPG2* (U70480); tomato *TAPG4* (U70481); peach fruit (X76735); Arabidopsis flower bud 2 (X72291); maize pollen (X57627); alfalfa pollen (U20431); Arabidopsis flower bud 1 (X73222); brassica pollen 1 (L19879).



**Fig. 3.** Cucumber genomic DNA gel-blot analysis of polygalacturonase. Genomic DNA (3  $\mu$ g / lane) was digested with *Hind*III (H), *Eco*RI (E) or *Xba*I (X). The blot was probed with the *CUPG1* and washed with 0.2 x SSPE and 0.1% SDS at 60  $^{\circ}$ C.



**Fig. 4.** Northern-blot analysis of PG mRNA; water loss and PG activity in cucumber fruit as affected by water stress, pre-treatment with MCP, ethylene or ABA treatment. Intact fruit were held in high humidity (97% RH) with or without ethylene (1000 ppm), or low humidity (40% RH) with or without pre-treatment with MCP for 24 hr at 25  $^{\circ}$ C. ABA (1000 ppm) solution was vacuum-infiltrated into fruit segments (10 cm long) cut from central part of the fruit at 400 mmHg for 1 min and control segment for ABA was treated with distilled water. The segments were held in humidified atmosphere for 24 hr at 25  $^{\circ}$ C. Each lane was loaded with 3  $\mu$ g poly (A)<sup>+</sup> RNA. Actin probe was used as an internal standard to confirm the integrity of each RNA preparation.



**Fig. 5.** Effect of MCP-pretreatment on stimulation of ACO activity by propylene in cucumber fruit. Vertical bars mean SE (n = 8).

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## キュウリ果実の水ストレス誘導ポリガラクトナーゼ遺伝子の発現解析

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## 摘 要

収穫後の水分損失ストレスに対するキュウリ果実の生理学的反応を調査した。低湿環境にさらした果実から、RT-PCR法を用いてポリガラクトナーゼをコードする遺伝子として *CUPGI* をクローニングした。RACE-PCR法によって全鎖長遺伝子の塩基および推定アミノ酸配列を決定したところ、*CUPGI* は435のアミノ酸から成り、既報のPG遺伝子に保存されている領域を含んでいた。この遺伝子は水ストレスおよび外生エチレンによって発現が誘導されたが、ABA処理に

は反応しなかった。水ストレスによる *CUPGI* mRNA の蓄積がエチレン作用の阻害剤であるMCPの前処理によって約半分に抑制されたことから、乾燥による *CUPGI* の発現は、水ストレスによる直接およびエチレンを介した間接的誘導機構の両者によって制御されているものと推測された。これらの結果から、収穫後のキュウリ果実の水分損失は単に物理的变化を引き起こすだけでなく、*CUPGI* の発現誘導を含む生理学的な反応につながるものと結論した。