液胞の機能分子の活性発現に関与する液胞プロセシング系の解析

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要旨

高等植物の液胞は，膨圧の維持，代謝産物の蓄積，細胞内成分の分解，貯蔵タンパク質及び生体防御タンパク質の蓄積という種々の機能果たす．この機能の発現のため，植物には2種類の液胞（タンパク貯蔵型液胞と分解型液胞）が存在しそれぞれに特異的な酵素や分子装置が使い分けられている．

液胞プロセシング酵素（VPE）はヒマの種子貯蔵タンパク質の前駆体を成熟型に変換する酵素として発見された．この酵素は，新規のシステインプロテアーゼで，アスパラギン及びアスパラギン酸残基を特異的に認識して切断するエンドペプチダーゼであることが示されている．その後，種々の植物よりVPEのホモログが単離されるにつれて，VPEには貯蔵組織型と栄養組織型に分けられることが明らかになった．さらに，ヒト，マウスなどの動物細胞よりVPEのホモログが単離され，液胞プロセシングの系が広く存在する可能性が示唆されている．そこで，本研究では，貯蔵組織および栄養組織のVPEの標的分子に注目し，そのプロセシング様式を解明するとともに各々の組織の液胞プロセシング系を分子レベルで明らかにすることを目指した．

貯蔵組織のVPEの標的分子である種子タンパク質は細胞内の粗面小胞体で合成され，直径が300 nm の輸送小胞であるPAC小胞を経て液胞に輸送される．PAC小胞の構成成分であるおよそ100 kDaのタンパク質であるPV100のcDNAクローニングよりPV100はシステインが特徴的に配置されているCxxxCモチーフを4つ持つCys領域，親水性のアミノ酸であるアルギニン，グルタミン，グルタミン酸に富むRE領域，種子貯蔵タンパク質であるビシリン領域から構成されていることが明らかになった．また，プロテインボディに含まれるタンパク質の解析により，PV100のそれぞれの領域が翻訳後に50 kDaのビシリンと5-10 kDaのCys領域及びRE領域由来の小さなペプチドにVPEによりプロセシングされることが明らかになった．Cys領域由来のC2にはトリプシンインヒビター活性が見られたが，C2の前駆体であるPV100にはトリプシンインヒビター活性が見られ

なかった．この結果はプロセシングを受けることによってC2の機能が発現することを示しており，VPEが種子の液胞タンパク質の機能発現を制御していることを初めて示すものであった．

栄養組織におけるVPEの標的分子はこれまでに明らかにされていない．そこで，プロテアーゼ活性の高い栄養組織の液胞におけるタンパク質の液胞プロセシング系にVPEがどの様に関与しているかを検討した．シロイヌナズナを用いて葉の傷害や老化によるVPEのmRNAが増えることが示されていたが，タンパク質レベルでも栄養組織のVPEが急激に増加することが明らかになった．また，シロイヌナズナのγVPEを酵母に発現させたところ，貯蔵組織のVPEと同様にアスパラギン特異的なエンドペプチダーゼであることが確認された．キウイのパパイン型システインプロテアーゼであるアクチニジンのC末端はアスパラギン残基でプロセシングされ，活性化することが知られている．乾燥ストレスで誘導されてくるシロイヌナズナのRD21Aとアクチニジンの一次アミノ酸配列の比較により，RD21Aもアスパラギン残基の後ろでプロセシングされると考えられた．実際に，RD21Aタンパク質の蓄積が葉の老化に伴って上昇してくること，γVPEが乾燥ストレスで誘導されてくることから，γVPEがRD21Aをプロセシングし活性化することが考えられ，プロテアーゼ活性の高い栄養組織の液胞においてもVPEがプロセシングに関わっていることが示唆された．

VPEは液胞の機能タンパク質の成熟化に関与し，ひいては液胞の機能発現を担う．そこで，VPEの発現調節機構を解析することによりこれまで明らかになっていなかった液胞の機能変換を明らかにすることができると考えた．種々の条件を試したところ，葉が傷害を受けたとき，γVPEの発現がおよそ12時間後から誘導されることが明らかになり，液胞が活発に働いていることが示唆された．また，傷害を受けたときに誘導されるプロテアーゼインヒビターやリポキシゲナーゼなどの液胞タンパク質がジャスモン酸により誘導されることが知られているが，γVPEはジャスモン酸に反応しなかった．このことは，傷害による新たな遺伝子発現の制御が行われていることを示唆していた．

以上のように，本研究ではVPEが種々のタンパク質の活性型への変換に必要であることが明らかになった．また，傷害におけるγVPEの発現は既知のシグナル伝達の解析に新たな知見を加えるものと期待される．

目 次

略　号

ABRC	: Arabidopsis Biological Research Center
BAPA	: α-N-benzoyl-DL-arginine-*p*-nitroanilide HCl
bp	: base pair(s)
BSA	: bovine serum albumin
CBB	: Coomassie brilliant blue
cDNA	: complementary DNA
CMTI	: *Cucurbita maxima* trypsin inhibitor
DW	: distilled water
EDTA	: ethylenediamine tetraacetic acid
EST	: expression sequence tag
GST	: glutathion *S*-transferase
kDa	: kilodalton
mRNA	: messenger RNA
MES	: 2-(N-morpholino) ethanesulfonic acid
min	: minute(s)
PAC	: precursor-accumulating
PAGE	: polyacrylamide gel electrophoresis
PBP	: pumpkin basic peptide
PBS	: phosphate-buffered saline
PCR	: polymerase chain reaction
PI	: proteinase inhibitor
PMSF	: phenylmethylsulfonylfluoride
PR	: pathogenesis related

PV : PAC vesicle

RACE : rapid amplification of cDNA ends

rbcS : ribulose bisphosphate carboxylase/oxygenase small subunit

RD : response to dehydration

SAG : senescence associated gene

SAR : systemic acquired resistance

SDS : sodium dodecyl sulfate

TBS : Tris-buffered saline

Tris : tris (hydroxymethyl) aminomethane

VPE : vacuolar processing enzyme

VSP : vegetative storage protein

その他核酸，アミノ酸の略号として一文字表記および三文字表記を用いた．

序 論

植物の液胞について

高等植物の液胞は，単位膜で囲まれたオルガネラであり，動物のリソソームに対応する．しかし，高等植物の液胞は動物や酵母の液胞にはない特徴がある．

植物は吸収した土壌や空気中の有害物質，細胞内で生じた不要物質を細胞外に放出せず，液胞内に蓄積する．植物細胞にとり有害な有機化合物はグルタチオン-*S*-トランスフェラーゼによりグルタチオンが付加され，液胞膜のグルタチオンポンプ (Lu et al., 1997)により液胞内に能動的に取り込まれる (Martinoia et al., 1993)．また，液胞内に蓄積した有害物質や不要物質の分解に関わる分解系の酵素の解析も進められつつある．

高等動物は，外敵から身を守る機構として免疫系をもっている．植物は免疫系をもっていないが，菌の感染や虫の食害に遭った場合，素早く液胞に抗菌性のタンパク質を蓄積し，それ以上食害を受けたり感染が広がらないように液胞に外敵から身を守る機構を備えている(Kauffmann et al., 1987, Kombrink et al., 1988, Graham et al., 1985a)．そのほかにも，植物はもともと独立栄養生物なので，種子には発芽の時に発芽の時に必要なタンパク質を合成する窒素，硫黄の供給源として，種子貯蔵タンパク質が液胞内に蓄積している (Shewry et al., 1995).

植物の液胞の種類

酵母や動物の液胞 (リソソーム)は主に分解機能しか持たないので，その構成成分も単純であると考えられる．しかし，植物の液胞は先に挙げたように種々の機能をもっている．植物の液胞には1) タンパク質貯蔵型液胞，2) 分解型液胞の二つの種類があり，植物の液胞が持つ様々な機能をそれぞれ分担している．

タンパク質貯蔵型液胞は，種子のプロテインボディに代表されるように，貯蔵のための小器官である．タンパク質貯蔵型液胞には2S albumin，11S globulin，7S globulin等の種子貯蔵タンパク質，フィチン酸のようなリン酸を多く含む有機化合物が蓄積する．また，Chitinase，Protease inhibitor等の病害や虫害に対する防御の役割を果たすタンパク質も蓄積する．よって，種子のタンパク質貯蔵型液胞は，発芽時のエネルギー源となる物質を貯蔵する役割と，種子を捕食者等から守る役割を備えている．一方，分解型液胞は，主に栄養組織にみられる．分解型液胞は，Central Vacuoleのように細胞の成長と共に大きくなり全体積の80-90%を占めるようになるが，そのタンパク質含有量は，全タンパク質の10%にすぎない．また，炭水化物やタンパク質の分解系の酵素に富むため (Nishimura and Beevers, 1979, Boller et al., 1979)，貯蔵タンパク質やサイトゾルから取り込んだ不要成分を分解する役割を果たしている．そのほかにも様々な代謝産物や有害物質が蓄積するため，植物の生育や環境適応能力の獲得に重要な役割を果たしている (Wink, 1993).

タンパク質貯蔵型液胞には，貯蔵組織特異的な水チャネルであるαTIP (TIP; Tonoplast intrinsic protein)がみられ (Johnson et al., 1989, 1990)，分解型液胞には栄養組織特異的な水チャネルであるγTIP (Hofte et al., 1992, Maeshima et al., 1992)が存在する．近年，この二つのTIPをマーカーにした解析より，栄養組織である根端の細胞にもタンパク質貯蔵型液胞と分解型液胞の双方が存在することが報告されている (Paris et al., 1996).

分解型液胞にもタンパク質が蓄積する時期がある．ダイズにおいて，傷害を受けると葉の液胞にVegetative storage protein (VSP)と呼ばれるタンパク質が蓄積することが知られている (Staswick, 1994)．VSPには，リポキシゲナーゼなどが含まれるが (Transbarger et al., 1991, Bell and Mullet, 1991)，あまりにも多量に

蓄積するため酵素としての役割以外に窒素源の貯蔵の役割も果たしているとも考えられている．また，トマトにおいて，傷害誘導性のプロテアーゼインヒビターも，葉の液胞に多量に蓄積することが報告されている (Graham et al., 1985a, 1985b)．最近，これらのタンパク質を蓄積する液胞はδTIPが多く含まれることが示され，分解型液胞とは多少異なることが示された (Jauh et al., 1998)．この液胞はデルタ型液胞と名付けられ，抵抗性タンパク質を分解型液胞のプロテアーゼから守るために分化するオルガネラであると思われる．

種々のストレスにおける液胞タンパク質の発現誘導

植物が病原菌に感染したとき，植物側が感染した病原菌に対する抵抗遺伝子をもっていた場合，一連の抵抗反応が引き起こされ(Yang et al., 1997, Shah, 1997)，液胞に抵抗性のタンパク質を蓄積する．この抵抗反応は全身獲得抵抗性 (SAR; Systemic acquired resistance)と局所獲得抵抗性 (LAR; Localized acquired resistance)の二つに分けられる．局所獲得抵抗性とは，菌が感染した部位における抵抗性で，細胞が自発的に死んでいく過敏感細胞死 (HR; Hypersensitive reaction)として観察される．また菌感染から遠い場所には菌に抵抗性を持つPR (Pathogenesis related)タンパク質が液胞に蓄積する．これらのタンパク質には，菌の細胞壁を分解すると考えられている，キチナーゼ，β-1,3-グルカナーゼなどが含まれる (Kauffmann et al., 1987, Kombrink et al., 1988)．菌感染時のSARを誘導する物質として，サリチル酸が同定されている (Klessig and Malamy, 1994, Delaney, 1997, Gaffney et.al., 1993, Delaney et al., 1994)．シロイヌナズナでも分解液胞型のVPEがサリチル酸により誘導されることより，菌感染により誘導される全身獲得抵抗性に何らかの影響を与えていると思われる．

植物が虫の食害により傷を受けたときは傷害に対するSARが起こる．トマ

ト (*Lycopersicon peruvianum*)のプロテアーゼインヒビターである (PI-I, PI-II)は傷害により誘導される(Graham et al., 1985a, 1985b). また, オオムギ (*Hordeum vulgare*)やシロイヌナズナ (*Arabidopsis thaliana*)では抗菌活性を持つThionin (Bohlman, 1994)やDefensin (Epple et al., 1997)が傷害により誘導される. これらの傷害誘導性のタンパク質は傷を受けた部位だけでなく全身的に誘導されて耐虫性を獲得すると考えられる (Koiwa et al., 1997). 傷害におけるSARを誘導する物質として, ジャスモン酸, エチレン, アブシジン酸などのホルモンが関与していることが明らかになっている. シロイヌナズナやダイズ (*Glycine max*)やトマト (*Lycopersicon esclentum*)において, 傷害を受けることによりジャスモン酸およびエチレンの合成が誘導されること (Creelman et al., 1992, O'Donnell et al., 1996, McConn et al., 1997), 傷害で誘導されてくるタンパク質がジャスモン酸でも誘導されてくることが示されている (Bell and Mullet, 1991, Pena-Cortes et al., 1993, Benedetti et al., 1995). また, シロイヌナズナ (*Arabidopsis thaliana*)やトマト (*Lycopersicon esclentum*)のジャスモン酸が合成されない突然変異体では虫害に対する抵抗性が弱まっていることが示されている (Howe et al., 1996, McConn et al., 1997).

アブシジン酸を合成できないトマト (*Lycopersicon peruvianum*)の突然変異体では傷害で誘導されるプロテアーゼインヒビターの発現がみられないこと (Herde et al., 1996, Pena-Cortes et al., 1996), トマト (*Lycopersicon esculentum*)やジャガイモ (*Solanum tuberosum*)の傷害誘導性の遺伝子がアブシジン酸に応答すること (Pena-Cortes et al., 1989, Birkenmeier and Ryan, 1998, Dammann et al., 1997)から傷害時の遺伝子発現におけるアブシジン酸の関与が示唆されている. 傷害におけるアブシジン酸のシグナルはジャスモン酸のシグナルとは異なっていることが示されているが(Lee et al., 1996, Dammann et al., 1997), まだ不明なところが

多い．そのほかにも，トマトにおいて低分子のペプチドであるSystemin，多糖類，膜電位の変化が傷害に対する全身的なPI-IIの発現に関与していることが示されており (Bishop et al., 1984, Pearce et al., 1991, McGurl et al., 1992, Wildon et al., 1992)，Sysitemin や多糖類に結合する受容体の解析が進められている．

植物が乾燥にさらされたときには水分を多く含む液胞が機能的に働くと考えられる．また，乾燥にさらされたときに発現してくるシロイヌナズナの遺伝子の中には，液胞膜の水チャネルであるγTIP (Yamaguchi-Shinozaki et al., 1992)や液胞に輸送されると考えられるプロテアーゼが存在する (Koizumi et al., 1993)．また，塩ストレスにより誘導されるタバコ (*Nicotiana tabacum*)のオスモチン様タンパク質，NP24はPRタンパク質の一つであるPR-5のホモログで (Zhu et al., 1993)，液胞に蓄積することが知られている．

液胞へのタンパク質の輸送とプロセシング

植物の栄養組織では，動物や酵母と同様にほとんどの液胞タンパク質は粗面小胞体上で合成され，直径が50nm程の輸送小胞によりゴルジ体を経由した後に液胞に運ばれると考えられている．実際，これらの証拠を示す小胞輸送の分子装置も見つかってきている．一方，植物の種子の登熟時には種子貯蔵タンパク質が粗面小胞体上で多量に合成される．そのため，液胞への輸送方法も異なっている．エンドウマメの種子の登熟期の貯蔵タンパク質の輸送には栄養組織に見られるクラスリン小胞が関与せず，直径50 nm程の電子密度の高い小胞により輸送されている (Hohl et al., 1996)．カボチャでは種子の登熟期にさらに大きな直径300 nmほどの電子密度の高い小胞が貯蔵タンパク質のタンパク質の輸送に関与している．この小胞は種子貯蔵タンパク質の前駆体を多量に含むためPAC(Precursor-accumulating)小胞と名付けられた (Hara-Nishimura et al., 1998)．種

子の登熟時には多量の液胞タンパク質が種子貯蔵タンパク質として合成されるため，輸送方法として大きなPAC小胞が関与していると考えられる．また，PAC小胞に含まれるタンパク質のプロ型前駆体はゴルジ体の輸送を阻害するモネンシンの影響を受けないこと，タンパク質貯蔵型液胞に小胞体由来のBipが蓄積していることより，登熟期の貯蔵タンパク質はゴルジ体を経由しないで貯蔵タンパク質に輸送されると考えられている (Hayashi et al., 1988, Hara-Nishimura et al., 1998).

多くの液胞タンパク質はプロペプチドを持った不活性型で合成され輸送過程では機能を発現せず，液胞に到達しプロペプチドの除去に伴い活性を発現することが知られている．酵母 (*Saccharomyces cerevisiae*)の液胞では，アスパラギン酸エンドペプチダーゼであるProteinase Aが液胞内の種々のタンパク質のプロセシングに関与している (Ammerer et al., 1986, Jones et al., 1982, Wooldford et al., 1986)．酵母のProteinase A (PrA)の活性がみられない*pep4* 変異株では他の液胞のプロテアーゼであるProteinase B (PrB)および，Proteinase C (CPY)，Alkaline phosophatase (ALP)をはじめとする液胞タンパク質の成熟型への変換が起こらない．一方，植物の液胞ではシステインエンドペプチダーゼである液胞プロセシング酵素(VPE，後述) やアスパラギン酸エンドペプチダーゼが種々のタンパク質のプロセシングに関与している．

一つの液胞タンパク質に多数のプロセシング酵素が関わり，多段階にプロセシングされることもある．酵母ではセリンエンドペプチダーゼである，PrBの活性がみられない*prb1* 株おいて，CPYは成熟型よりも少し大きい分子量で確認され，CPYの完全な成熟型への変換にはPrBの働きが必要であることを示している (Mechler et al., 1987, Hirsch et al., 1992)．しかし，PrAによるCPYの部分的なプロセシングでもCPYの活性は発現する．また，ヒト(*Homo sapience*)のPrA

ホモログであるCathepsin DはCathepsin Bの最終的なプロセシング部位の14アミノ酸残基手前で切断するが，生じたCathepsin Bにはプロテアーゼ活性が存在するようになる (Takio et al. 1983)．これらのことは少なくとも最終的な形を決定するプロセシング酵素と活性化に必要なプロセシング酵素の二種類が存在することを示している．

植物の液胞のプロテアーゼは数が多いので液胞タンパク質の多段階プロセシングも予想される．実際，ダイズ (*Glycine max*)種子のプロテアーゼであるP34 (Kalinski et al., 1992)，ケツルアズキ (*Vigna mungo*)の実生に発現するプロテアーゼであるSH-EP (Yamauchi et al., 1992)の成熟型へのプロセシングは多段階的で，幾つかの中間体が存在することが示されている．

液胞プロセシング酵素(Vacuolar processing enzyme)

植物の液胞のプロセシング酵素は種子貯蔵タンパク質の成熟化を研究する過程で明らかになった．種子の登熟期には貯蔵タンパク質が盛んに合成され，タンパク質貯蔵型液胞へと蓄えられる．これらのタンパク質はプロ型前駆体として合成され，液胞に運ばれた後に成熟型に変換される．当初は個々の貯蔵タンパク質に対して独立の酵素が働くと考えられ，ヒマのRicin (Harley and Load, 1985)，カボチャの11S globulin (Hara-Nishimura et al., 1987), ダイズ (*Glycine max*)のGlycinin (Scott et al., 1992)などの前駆体をプロセシングするタンパク質が別々に解析されてきた．しかし，ヒマの11S globulinをプロセシングする酵素は他の貯蔵タンパク質の前駆体も成熟型に変換することが示され (Hara-Nishimura et al., 1991)，一種類の酵素が複数の貯蔵タンパク質をプロセシングすることが明らかとなった．当初，この酵素はアスパラギン酸プロテアーゼであると考えられたが，最終的にヒマよりとられた酵素はシステインプロテアーゼであり，液

胞プロセシング酵素 (VPE; Vacuolar processing enzyme)と名付けられた．この酵素はヒト住血吸虫 (*Schistosoma mansoni*)のシステインプロテアーゼ (Sm32) (Klinkert et al., 1989)と相同性を示し，広く知られているパパイン型のシステインプロテアーゼと異なるファミリーを形成している (Hara-Nishimura et al., 1993b)．その後，カラスノエンドウ(*Vicia sativa*) (Becker et al., 1997)およびタチナタマメ(*Canavalia ensiformis*) (Abe et al., 1993)より同様の酵素が単離され，アスパラギンやアスパラギン酸を特異的に認識して働くエンドペプチダーゼであることがことが示された．近年になりヒト (*Homo sapience*)，マウス (*Mus musculus*)にもVPEのホモログが存在することが確認され (Chen et al., 1997, 1998)，VPEによる液胞プロセシングの系が広く存在していることが示唆される．

植物より同定されているVPEホモログの一次構造や遺伝子の発現様式の比較より，VPEは栄養組織型と貯蔵組織型に分類されることが明らかになった．実際，シロイヌナズナ (*Arabidopsis thaliana*)より，栄養組織型と貯蔵組織型両方のホモログのcDNAがクローニングされ，VPEがそれぞれタンパク貯蔵型液胞と分解型液胞で使い分けられていることが示された (Kinoshita et al., 1995a, 1995b)．VPEホモログの発現制御の解析が進むにつれ，液胞の機能が特異的に変化することが示唆された．特に，シロイヌナズナの栄養組織型のγVPEやαVPEの発現は，老化や傷害で誘導されてくること，バレンシアオレンジ (*Citrus sinensis*)の栄養組織型VPE (Cit-vac)がエチレンで誘導されてくることが明らかになり (Alonso and Granell, 1995)，これまでに知られていなかった傷害や老化における液胞の機能変換が起こっていることを示唆している．

本研究では第一章でカボチャの種子の登熟期におけるVPEの働きを基質となるタンパク質分子の解析より明らかにした．また，第二章，第三章ではシロイヌナズナの栄養組織に発現する液胞タンパク質の発現パターンやプロセシン

グのメカニズムを解析した．本研究から，液胞の機能変換の調節機構に対する分子生物学的なアプローチに新しい知見を加えることができたと考えている．

第1章　タンパク貯蔵型液胞における液胞プロセシング系の解析

種子貯蔵タンパク質は細胞内の粗面小胞体で合成され，PAC小胞を経て液胞に輸送される．本章では，PAC小胞の構成成分であるPV100の構造解析およびPV100由来のペプチドの解析より，(1) PV100がシステイン領域，RE領域，ビシリン領域から構成されていること，(2) PV100のから複数の液胞機能分子がVPEのプロセシングにより生ずること，(3) システイン領域由来のペプチドは前駆体PV100にはないトリプシンインヒビター活性を有することおよび(4)インヒビターの活性化にVPEによるプロセシングが関与していることを明確にした．以上の結果は，VPEが種子の液胞機能タンパク質の活性発現を制御していることを示している．

材 料 と 方 法

実験材料

カボチャ栽培種，(*Cucurbita maxima* cv. Kurokawa amakuri nankin)を実験材料として用いた．カボチャの登熟子葉は，夏期農場で育てた各生育段階のカボチャ果実より採取した．

オルガネラの単離

PAC小胞とタンパク貯蔵型液胞の一つであるプロテインボディの単離は，Shimada ら (1994, 1997)および Hara-Nishimura ら (1993a)の方法に従った．調製したプロテインボディからのマトリクス画分の単離は Inoue ら (1995a, 1995b)の

方法に従った.

免疫電子顕微鏡観察

試料の作製はHara-Nishimuraら (1993a)の方法に従って行った．カボチャの登熟子葉より単離したPAC小胞を固定液 (4% paraformaldehyde, 1% glutaraldehyde, 0.05 M sodium-cacodylate, pH 7.4, 0.06 M sucrose)に浸し，室温で1時間固定した．子葉組織の場合は，固定液が良く浸透するように組織を1時間減圧浸透処理した後に1 mm角以下に細切し，新しい固定液でさらに2時間処理した．上記の緩衝液で洗浄した後，ジメチルホルムアミド濃度勾配系列により-20 ℃で脱水した．試料はLR-White樹脂 (London Resin)に包埋し，紫外線重合装置 (Dohan EM)を用いて重合させた．ミクロトーム (Reichrt Ultramicrotome)で超薄切片を作製し，ニッケルグリッドにマウントした．切片をブロッキング液 (1% BSA in PBS)で室温で1時間処理した後，ブロッキング液で100倍希釈した各抗血清と4℃で一晩反応させた．PBSで洗浄後，切片はブロッキング液で20倍希釈したプロテインA-金コロイド液 (Amersham Pharmacia Biotech)で室温で30分間処理した．切片はDWで洗浄し，4%酢酸ウランとクエン酸鉛で染色した．観察は染色後切片を透過型電子顕微鏡 (1200EX, JEOL)を使用して80 kVで行った.

SDS-PAGEとウエスタンブロッティング

SDS-PAGEはLaemmli (1970)の方法に準じて行った．タンパク質試料は試料溶解液 (2% SDS, 50 mM Tris-HCl, pH 6.8, 5% 2-mercaptethanol, 0.1% BPB, 10% glycerol)に懸濁し，95℃で3分間熱処理した．その後，アクリルアミドゲルにロードした．泳動後，ゲル中のタンパク質はナイロン膜 (GVHP membrane, Millipore)に電気的に転写した．ゲルを転写用液 (100 mM Tris-glycine, pH 6.8,

20% (v/v) methanol)に浸し10分間浸透した後，同じ溶液で処理したナイロン膜とろ紙の間に挟んだ．ゲル中のタンパク質はセミドライブロッター装置 (Bio Craft)を用いて2 mA/cm^{2}の条件で電気的にナイロン膜に転写した．

タンパク質が転写されたナイロン膜は，3%スキムミルクを含むTBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween20)で1時間ブロッキング処理をした．その後，1/1000に希釈したPV100抗体と 3% (w/v)スキムミルクを含むTBS-Tで一晩振とうし，TBS-Tで5分4回洗浄した．その後1/2000に希釈したアルカリフォスファターゼ結合ヤギ抗ウサギIgG抗体 (Cappel)を含むTBS-Tで1時間浸透した．ナイロン膜を5分2回洗浄した後，発色用緩衝液 (50 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$)で5分2回振とうした．アルカリフォスファターゼの基質であるBCIP (5-bromo-4-chloro-3-indolylphosphate) (50 µg/ml)とNBT (nitroblue tetrazorium) (150 µg/ml)を含む発色用緩衝液中でナイロン膜を振とうし，反応するタンパク質を検出した．

N末端アミノ酸解析

PAC小胞のPV100と，プロテインボディのVicilinのアミノ酸配列を決定するために，単離したPAC小胞とプロテインボディのマトリクス画分をSDS-PAGEにかけ，タンパク質をナイロン膜に電気的に転写した．その後，膜をCBB染色し，PV100およびVicilinに相当するペプチドを切り出した．切り出した膜よりアミノ酸シーケンサー(Model 492, Applied Biosystems)を用いてEdman法により配列を解析した．

また，PV100のアミノ酸配列を決定するために，まず，PAC小胞をSDS-PAGEにかけ，CBB染色の後100 kDaのペプチドを切り出した．切り出したタンパク質はClevelandら(1977)の方法にしたがい，V8 protease (Sigma)を用いて消化

し，SDS-PAGEの後，ナイロン膜にタンパク質を転写し，アミノ酸シーケンスを行った．

cDNAの単離とシーケンス

PV100のアミノ酸配列をもとに4種類のオリゴヌクレオチド

1F (5'-GG(A/C/G/T)GC(A/C/G/T)GG(A/C/G/T)GT(A/C/G/T)GA(C/T)CA-3'),

2F (5'-CA(C/T)GA(C/T)GG(A/C/G/T)TG(C/T)GT(A/C/G/T)-3'),

3R (5'-GG(A/G/T)AT(A/C/G/T)GTCAT(A/C/G/T)AC(A/G)TC-3'),

4R (5'-TA(G/A)TC(T/C)TT(G/A)TC(T/C)TT(G/A)AA(T/C)TC(A/C/G/T)CC-3')

をデザインし，DNA合成機 (Model 394, Applied Biosystems)を用いて化学合成した．まず，カボチャの登熟子葉より作製されたプラスミドcDNAライブラリーを鋳型として1Fと4Rを用いPCRを行った．得られた1454 bpのDNA断片を鋳型として2Fと3RでPCRを行い，1340 bpのDNA断片を得た．1340 bpのDNA断片をT-ベクター; pBluescript-SK+ (Stratagene)にクローニングし，塩基配列を確認した．制限酵素*Eco* RI/*Hin* dIIIでクローニングした断片を切り出し，ランダムプライマー法によりアイソトープラベルしプローブとして使用した．標識にはMegaPrime (Amersham Pharmacia Biotech)を用い，[α-^{32}P] dCTPで行った．得られたポジティブクローンを常法に従いシーケンスを行ったところ，人工的に付加されたと思われる25S rDNA配列が逆向きに結合していた．また，開始コドンであるATGがPV100のコード領域の5'末端側に含まれていなかった．そこで，5'-RACE-Kit (Takara)を使用し5'RACE (Rapid amplification of cDNA ends)を行い，全長配列を決定した．シーケンスはDye Primer Cycle Sequence Kit (Applied Biosystems)を使用してDNA シーケンサー (Model 377, Applied Biosystems)を用いて解析した．

プロテインボディに含まれるペプチドの単離

調製したプロテインボディマトリクス画分より逆層クロマトグラフィーにより10 kDa以下のペプチドを分離した．プロテインボディマトリクス画分に30%飽和になるようにammonium sulfateを加え，200,000 x *g* で遠心した．その後上清に100%飽和になるようにammonium sulfateを加え，200,000 x *g* で遠心した．沈殿を25 mM sodium acetate, pH 5.5緩衝液に懸濁し，AKTA (Amersham Pharmacia Biotech)およびSMART (Amersham Pharmacia Biotech)を用いて低分子のペプチドを分離した．カラムはμRPC C2/C18 ST 4.6/100 (Amersham Pharmacia Biotech) (AKTA)およびμRPC C2/C18 PC3.2/3 (Amersham Pharmacia Biotech) (SMART)を用いた．緩衝液A (0.065% trifluoroacetic acid in DW)でカラムを平衡化し，試料を添加した後に緩衝液B (0.05% trifluoroacetic acid in acetonitrile)の連続濃度勾配によりペプチドを溶出した．流速は200μl/min (SMART)および500μl/min (AKTA)で行った．

質量スペクトログラフィー解析

プロテインボディマトリクス画分から回収したペプチドの正確な質量を決定するため，逆層クロマトグラフィーで分離したペプチドの質量スペクトログラフィー解析を行った．計測にはAPI 300 Triple Quadrupole Mass Spectrometer (PE SCIEX)を用い陽イオンを解析した．試料は0.1% formic acid, 50% acetonitrile に可溶化させ，3μl/minで射出した．Sprayerは4.5 kV，Orifice potential は25 Vに調節し，試料をイオン化させた．

トリプシンインヒビター活性の測定

プロテインボディマトリクス画分から精製したC2ペプチドおよびPAC小胞より精製したPV100を用い，トリプシンインヒビター活性をCechova (1976)の方法に従い測定した．トリプシン活性はBAPA (α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide)を用いた．C2およびPV100を10 μgのTrypsinを含んだ 676 μlの0.1 M Tris-HCl, pH 8.0, 25 mM $CaCl_2$と共に室温で30分間インキュベートした．その後，333 μlのDWで1 mg/mlに希釈したBAPAを加え，さらに30分間室温でインキュベートした後に，100 μlのacetic acidを加えTrypsinの反応を止め405 nmの吸光度を測定した．C2のモル濃度は280 nmを測定し，一次配列から計算したモル吸光係数を基に決定した．PV100のモル濃度はタンパク質の濃度より決定した．

ＰＶ１００の単離

カボチャの登熟子葉から単離したPAC小胞を3 mlの1 M NaCl, 10 mM Tris-HCl, pH 7.5 に可溶化した．超音波処理装置 (Model 450D, Branson)を使用し，Output 3，50% Cycleで30秒間超音波処理した後，21,000 x *g*で5分遠心し，不溶性の膜画分を取り除いた．その後，5-20%のショ糖密度勾配中で10,000 x *g*，17時間遠心し，沈殿を回収した．この遠心により，PV100のみ沈殿に回収される．回収した沈殿は，SDS-PAGE後CBB染色し，PV100が精製されていることを確認した．

ＶＰＥの精製

ヒマ種子よりプロテインボディを単離し，Con-A Sepharose (Amersham Pharmacia Biotech)とMono S (Amersham Pharmacia Biotech)を使用してHara-Nishimura ら(1993b)の方法に従ってVPEを精製した．

VPEによるPV100の*in vitro*プロセシング

カボチャより単離したPAC小胞を50 mM sodium acetate pH 5.5に懸濁し，ヒマ種子より精製したVPEと15時間，37℃で反応させ，反応産物をSDS-PAGEにより分離した．分離したタンパク質はナイロン膜に電気的に転写し，Vicilinに相当するペプチドを切り出した．切り出した膜はPyroglutamyl aminopeptidase (Boehringer Mannheim)で処理し，アミノ酸シーケンサー(492型，Applied Biosystems)を用いてEdman法により配列を解析した．

結　果

PV100はPAC小胞のタンパク質である

植物の液胞タンパク質は粗面小胞体で合成され，液胞まで小胞輸送される．大量に合成される種子貯蔵タンパク質の場合は直径がおよそ300 nmのPAC小胞が直接液胞への輸送小胞としての役割を果たす(Hara-Nishimura et al., 1998)．そこで，カボチャの登熟種子よりPAC小胞を単離し，含まれる貯蔵タンパク質の前駆体を解析した．

単離したPAC小胞をSDS-PAGEに供し，含まれるタンパク質をCBB染色により確認した (Fig. 1A)．Hara-Nishimuraら (1993a,1998)が報告しているように，PAC小胞には種子貯蔵タンパク質である11S globulinや2S albuminの前駆体が蓄積している．PV100はこれらの種子貯蔵タンパク質の次に多い構成成分として認識される．そこで，PAC小胞より精製したPV100を用いて抗体を作製し，ウエスタンブロッティングと免疫電顕を行った．ウエスタンブロッティングより作製した抗体がPV100特異的であることが示された．単離したPAC小胞に対して抗PV100抗体を用いて免疫電顕を行ったところ，金粒子のシグナルはPAC小胞に一様に強くみられ (Fig. 1B)，PV100はPAC小胞のタンパク質であることが確認された．

PV100は3つの領域より構成されている

PAC小胞に含まれるPV100のcDNAをクローニングするために，SDS-PAGEにより分離したPV100のペプチドを切り出し，N末端アミノ酸シーケンスを決定した．さらに，PV100をV8 proteaseにより消化し，分解産物のN末端アミノ酸シーケンスを決定した．その結果を基に，オリゴプライマーを作製し，cDNA

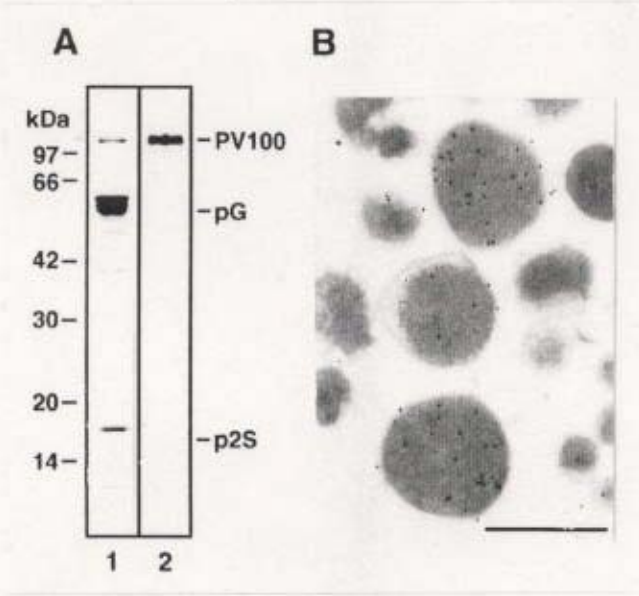


Fig. 1. PV100 is one of the major proteins in the PAC vesicles from maturing pumpkin seeds.

(A) Isolated PAC vesicles were subjected to SDS-PAGE and subsequent staining with Coomassie blue (lane 1) or immunoblot with anti-PV100 antibodies (lane 2). pG and p2S represent proprotein precursors of 11S globulin and 2S albumin, respectively. The molecular mass of each marker protein is given on the left in kDa. PAC vesicles were isolated from the cotyledons at the middle stage of seed maturation of pumpkin.
(B) Immunogold labeling of the isolated PAC vesicles with anti-PV100 antibodies. Bar = 500 nm.

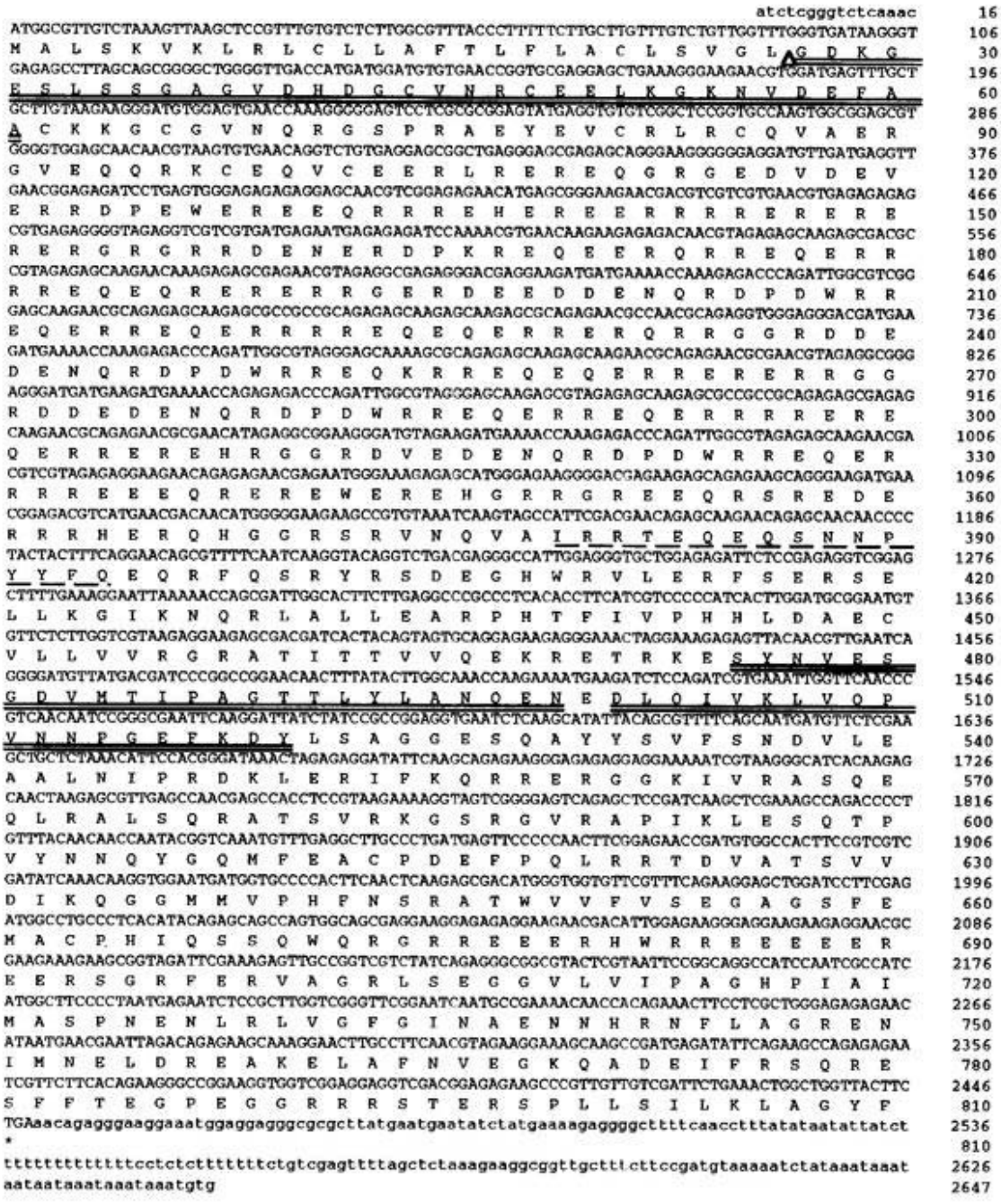


Fig. 2. cDNA and deduced amino acid sequences of PV100.

Isolated cDNA encodes a 97,310-Da protein of 810 amino acids, which consists of a hydrophobic signal peptide followed by the PV100 sequence. The N-terminal sequence and two internal sequences of PV100 that were determined are indicated by double-underlines. An open triangle indicates a cleavage site of a signal peptide. A dotted line indicates the N-terminal sequence of the vicilin-like protein from dry seeds. The nucleotide sequence has been submitted to the DNA Data Bank of Japan (DDBJ) with the accession number AB019195.

ライブラリーに対してPCRを行ったところ，1340 bpのDNAが増幅された．決定した塩基配列より増幅されたcDNAはPV100をコードすることがわかったので，この断片をプローブとしてスクリーニングを行った．その結果，およそ5 kbpのcDNAインサートを持つクローンを得た．このクローンは，5'末端に人工的に付加されたとみられる25S rDNA配列をもち，開始メチオニンが見つからなかった．そこで，5'RACEによりPV100の全長配列を決定した．

得られたクローンの推定アミノ酸配列にはPV100のアミノ酸配列が含まれていた (Fig. 2)．また，プロテインボディのVicilinのN末端アミノ酸配列も含まれており，PV100は，Vicilinの前駆体であることが明らかになった．N末端には疎水性アミノ酸に富むシグナル領域があり，von Heijne (1986)により決定されているシグナル切断部位のアミノ酸配列が保存されていた．

PV100は50 kDaのビシリン様領域の他に11 kDaのシステイン領域，34 kDaのRE領域の3つの領域より形成されていた (Fig. 3)．システイン領域は，二つのシステインが3つおきに配置されているCxxxCモチーフが4つ存在している．ホモロジー検索の結果，ビシリン様領域は植物に広く保存されている種子タンパク質であるビシリン (7Sグロブリン)と相同性があることが分かった．各植物のビシリンの前駆体を比較したところ，エンドウマメ (*Pisum sativum*)のVicilin (Watson et al., 1988)，ダイズ (*Glycine max*)のβ-Conglycinin-β (Harada et al., 1989)，ナタマメ (*Canavalia ensiformis*)のCanavalin (Ng et al., 1992)と30-35%の相同性があった．これらの植物のビシリンはシグナル領域のすぐ後にビシリン様領域が始まるが，カカオ (*Theobroma cacao*)のVicilin (McHenry et al., 1992)とアプランドワタ (*Gossypium hirsutim*)のα-Globulin-A (Chlan and Fritz, 1987)には システイン領域があり，それぞれ6つと4つのCxxxCモチーフがビシリン様領域の前に存在していた．また，ソバ (*Fagopyrum esculentum*)より同定されたトリプシンイン

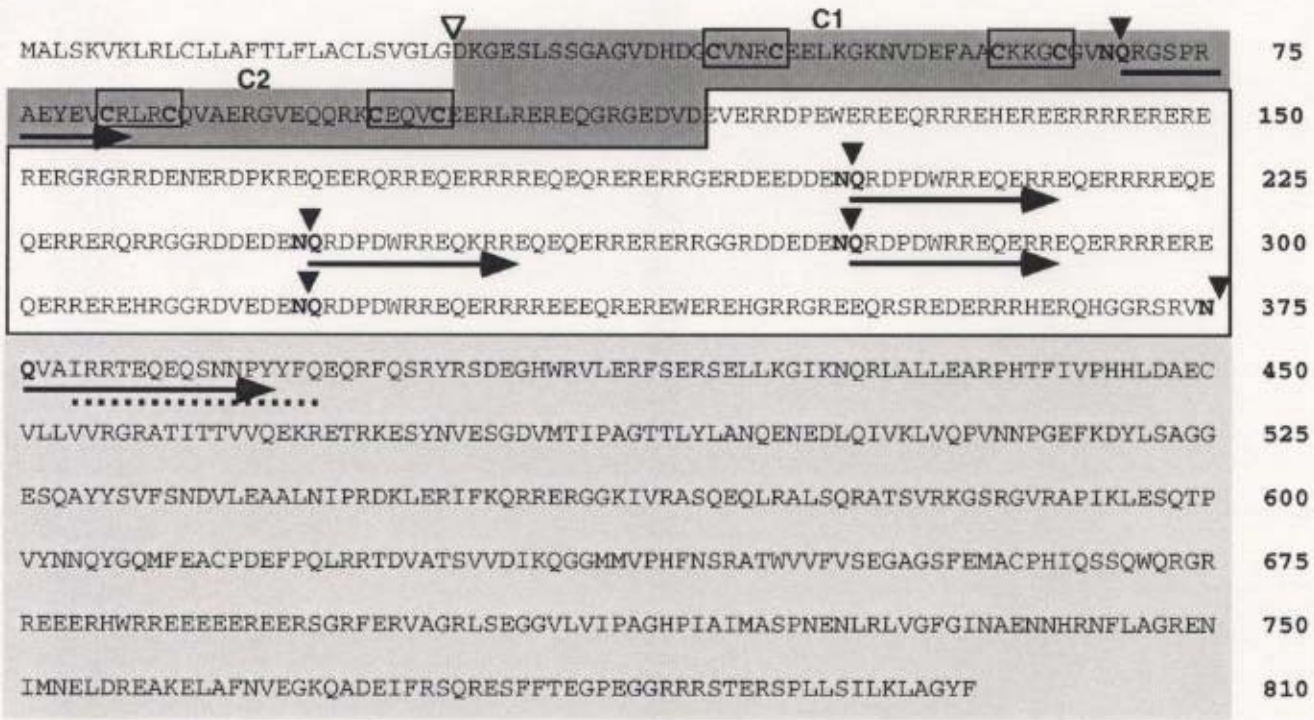
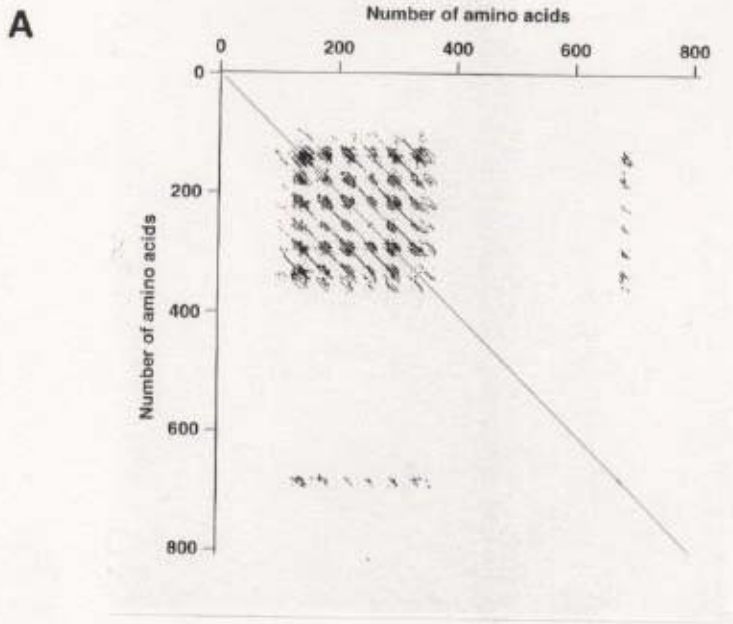


Fig. 3. A characteristic structure of PV100.

The PV100 sequence was divided into three domains: an 11-kDa Cys-rich domain (indicated by shading) with four CxxxC motifs (enclosed by small boxes), a 34-kDa Arg/Glu-rich domain (enclosed by a large box), and a 50-kDa vicilin-like domain (indicated by shading). An open triangle indicates a cleavage site of a signal peptide. Arrow indicates the determined N-terminal sequences of PV100-derived mature proteins that had been digested by pyroglutamate aminopeptidase (see Figs. 7 and 9), and a dotted line indicates the N-terminal sequence of the vicilin-like protein from dry seeds. Bold-faced NQ (Asn-Gln) stretches with a closed triangle represent post-translational processing sites to produce multiple seed proteins, each with a pyroglutamate at its N-terminus.



B

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RE1   RRDPEWEREEQRRREHEREERR--RREREREREREER---GRGRR-DEN          161
RE2   ERDPKREQEERQRREQERRRRE--QEQREREERRG---ERDEEDDEN           202
RE3   QRDPDWRRE-QERREQERRRREQEQERREREQRRG---GRDDE-DEN           243
RE4   QRDPDWRRE-QKRREQE-------QERREREERRG---GRDDE-DEN           277
RE5   QRDPDWRRE-QERREQERRRREREQERREREHRG---GRDVE-DEN            318
RE6   QRDPDWRRE-QERRRREEEQRER-EWEREHGRRGREEQRSRE-DER            361
```

C

Fig. 4. Homology plot of PV100 and an amino acid alignment of the six homologous repeats in the Arg/Glu-rich domain.

(A) A homology plot is performed by the PAM-250 algorithm with a window of 10 residues. Each pair of windows that exhibits more than 35% identity in amino acids is indicated by a dot in the matrix. Six homologous repeats were found in the Arg/Glu-rich domain.
(B) Asn-Gln/Glu bonds separate the Arg/Glu-rich domain into six repeats (see Fig. 10). The six Arg/Glu-rich repeats that were designated RE1 to RE6 in order from the NH_2 terminus were aligned. Numbers on the right side of each sequence refer to the positions of the amino acids starting from the initiation Met.
(C) The mature RE3 peptide (see Fig. 7B) was aligned with the sequence of pumpkin basic peptide, where the second and the third possible amino acids are also shown, as reported by Naisbitt et al. 1988. Both peptides are composed of 36 amino acids, as indicated on the right side of each sequence. Boxes enclose identical amino acids and shading indicates homologous amino acids.

ヒビター (BWI-2b)にも2つのCxxxCモチーフが存在している (Park et al., 1997). しかし，CxxxCモチーフ付近でのアミノ酸配列は保存されておらず，10%以下の相同性であった.

RE領域はそれぞれ37 mol%のアルギニンと，27 mol%のグルタミン酸からなり，親水性アミノ酸に富む領域であった．この領域はカボチャのPV100のみに存在し，他の種類のビシリン前駆体にはみられなかった．ホモロジープロットより，この領域は6つの繰り返し配列 (RE1-6)から構成されていることが明らかになった (Figs. 4A, B)．この配列は，Naisbittら(1988)によって同定されたカボチャ (*C. maxima* cv. Mexican-papitas)の塩基性ペプチド (PBP; Pumpkin basic peptide)と相同性があり，特にRE3はPBPと相同であると考えられた (Fig. 4C).

PV100由来のタンパク質の細胞内局在性

先の結果より，プロテインボディ内にはPV100由来のビシリン様タンパク質やペプチドが存在することが示唆された．そこで，これらのタンパク質の細胞内局在性を確認するために，抗PV100抗体を用いてカボチャの登熟子葉に対して免疫電子顕微鏡観察を行った (Fig. 5A)．カボチャ子葉には，電子密度の高いPAC小胞と，11S globulinからなるクリスタロイドを持つプロテインボディがみられる．金粒子のシグナルは，小胞体，PAC小胞，プロテインボディマトリクスにみられる．このことより，PV100由来のタンパク質がプロテインボディに蓄積することが明らかになった.

PV100から切り出されたタンパク質の同定

プロテインボディに運ばれたPV100は成熟型に変換されると考えられる．そこで，カボチャ種子よりプロテインボディを単離し，抗PV100抗体を用いて

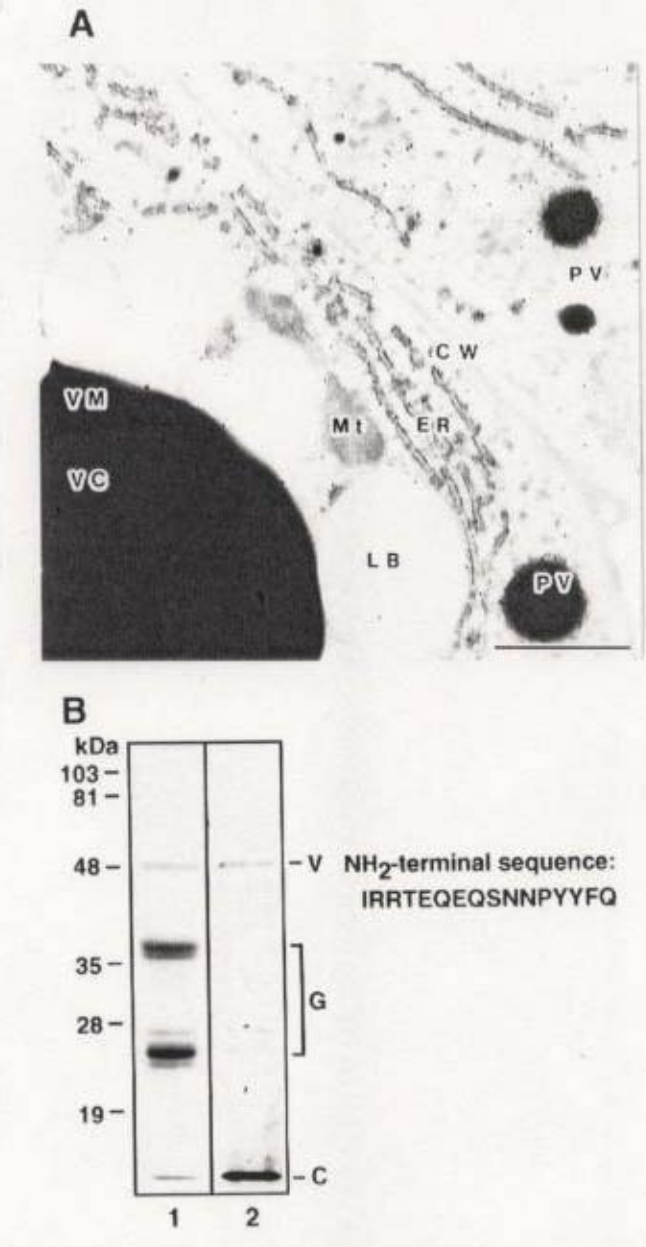


Fig. 5. PV100-derived proteins are localized in protein-storage vacuoles in pumpkin seeds.

(A) Immunoelectron micrograph of maturating pumpkin seeds after staining with anti-PV100 antibodies. Gold particles were distributed in the PAC vesicles (PV), the matrix region (VM) of protein-storage vacuoles and ER. VC, vacuolar crystalloid composed of 11S globulin; Mt, mitochondrion; LB, lipid body; CW, cell wall. Bar = 1 μm.

(B) Isolated protein-storage vacuoles (protein bodies) from dry pumpkin seeds were subjected to SDS-PAGE and subsequent staining with Coomassie blue (lane 1) or immunoblot with anti-PV100 antibodies (lane 2). PV100-derived proteins, the 50-kDa vicilin-like protein (V) and ~6-kDa C2 peptide (C), were detected on the blot. The determined N-terminal sequence of the 50-kDa vicilin-like protein is shown. G represents 11S globulin. The molecular mass of each marker protein is given on the left in kDa.

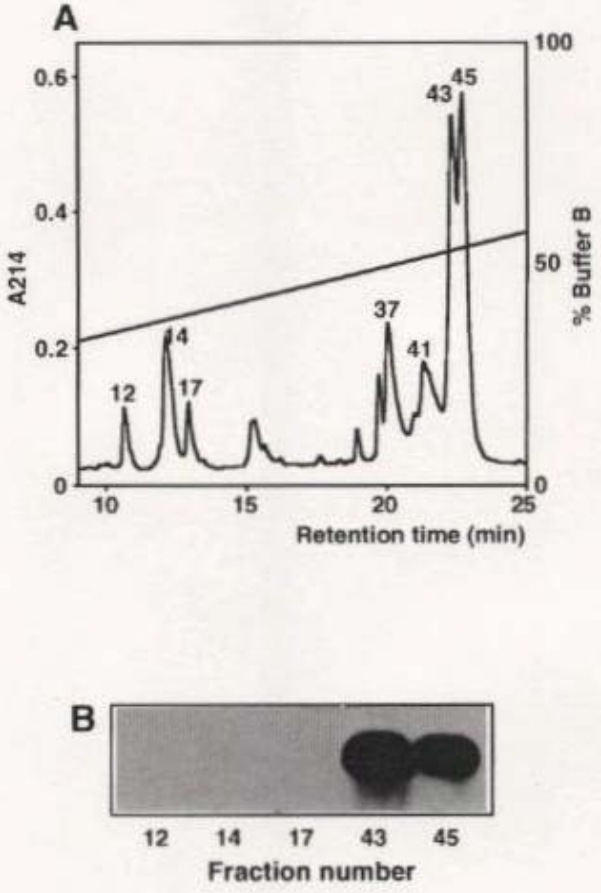


Fig. 6. An HPLC profile of PV100-derived peptides from the protein-storage vacuoles.

(A) Soluble fraction of the protein-storage vacuoles that contained the PV100-derived peptides was applied to a reverse-phase column. Elution was carried out with a gradient starting from 0.065% trifluoroacetic acid in distilled water to 0.05% trifluoroacetic acid in acetonitrile. Chromatography was monitored in terms of absorbance at 214 nm.
(B) Immunoblot analysis of each peak fraction with anti-PV100 antibodies.

ウエスタンブロッティングを行ったところ, 50 kDaのVicilinに反応した (Fig. 5B). このペプチドを切り出し，アミノ酸シーケンスを行ったところ，PV100の後半部分の配列が同定された (Figs. 2, 3). また，泳動先端付近の10 kDa以下のペプチドにも反応した．PV100のシステイン領域，RE領域の6つの繰り返し配列にはVPEにより切断されると考えられるAsn-Gln (Glu)配列が存在しており (Fig. 3). プロテインボディ内にプロセシングされたPV100のシステイン領域，RE領域由来の小さなペプチドが存在している可能性が考えられた．そこで，プロテインボディのマトリクス画分を出発材料として，PV100由来のペプチドの単離を試みた (Fig. 6). マトリクス画分の30-100%硫安画分を回収し，逆層クロマトグラフィーにかけ，分離されたペプチドのアミノ酸配列を決定した．画分37，41は既知のトリプシンインヒビター (CMTI) (Wieczorek et al., 1985)であったが，画分12，14，17，43，45のN末端はブロックされていた．そこで，Pyroglutamyl aminopeptidaseを作用させ，N末端のピログルタミン残基をはずした後にアミノ酸配列を決定したところ，それぞれ，画分12，14，17はRE4，RE3，RE5に相当し，画分43，45はC2に相当することが明らかとなった．さらに，これらのペプチドの構造を解明するために，質量スペクトログラフィーによる解析を行った (Fig. 7). その結果，RE4，RE3，RE5，C2ともにC末端はアスパラギン酸残基であった．また，C2の4つのシステインはそれぞれジスルフィド結合していることが明らかとなった．このことより，これらのペプチドは少なくとも二ヶ所でプロセシングされていると考えられた．また，RE3，RE4，RE5ペプチドの推定等電点 (pI)は，それぞれ11.90，11.54，10.20とアルカリ側に偏っていた

PV100由来のC2ペプチドはトリプシンインヒビター活性がある

次に，プロテインボディに蓄積しているPV100由来のペプチドの機能が問

A

Fraction number	Observed mass	Name of peptide	Sequence in (B)	NH_2 terminus	Number of disulfide bonds	Theoretical mass
12	3876.52±0.71	**RE4**	d	<Q	0	3877.00
14	4888.00±0.50	**RE3**	c	<Q	0	4888.03
17	5242.17±0.90	**RE5**	e	<Q	0	5241.34
43	5615.54±0.70	**C2**	a	<Q	2	5615.02
45	5829.90±0.80	**C2**	b	<Q	2	5829.23

B

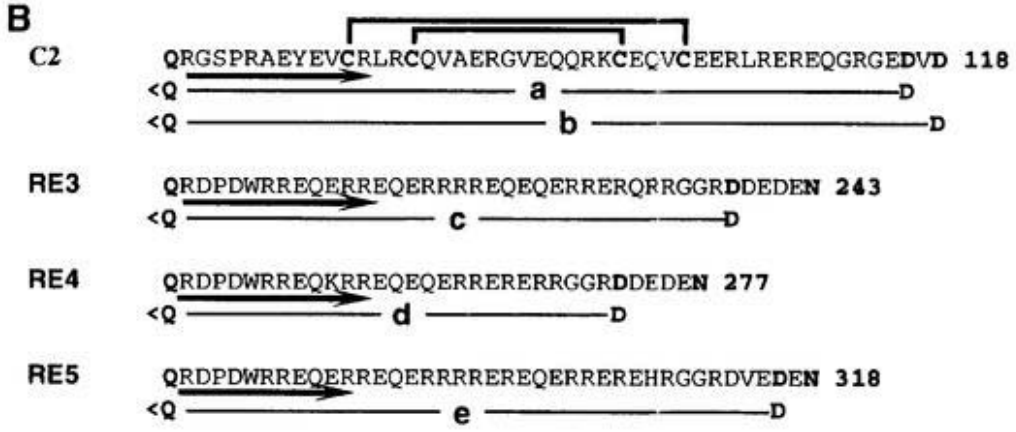


Fig. 7. Molecular structures of PV100-derived peptides from the protein-storage vacuoles.

(A) Molecular masses of the peptides in each HPLC fraction, as shown in Fig. 6, were determined by mass spectrometry. Theoretical molecular masses of RE3, RE4 and RE5 that had a pyroglutamate (<Q) at their NH_2 termini are consistent with the observed values. The number of disulfide bonds in the C2 peptide was determined to be 2.
(B) Primary structures of peptide components of fractions 12, 14, 17, 43 and 45 were determined to be sequence d (RE4), sequence c (RE3), sequence e (RE5) and sequences a and b (C2), respectively. The determined N-terminal sequences after digestion by pyroglutamate aminopeptidase are indicated by arrows below the respective sequence. Numbers on the right side of each sequence refer to the positions of the amino acids starting from the initiation Met of PV100. The disulfide bridges were deduced from the data of buckwheat trypsin inhibitor that exhibits a similar characteristic to the C2 peptide and has two CxxxC motifs and two disulfide bridges (Park et al. 1997).

題となった．C2ペプチドと低い相同性があるペプチドとして，ソバより単離，精製されたトリプシンインヒビター(BWI-2b)が見つかった (Park et al., 1997). BWI-2bはCxxxCモチーフを二つ持つおよそ5 kDaほどのペプチドで，4つのシステインがそれぞれジスルフィド結合しており，構造的にもC2と似ていると思われた．そこで，C2ペプチドのトリプシンインヒビター活性を検討した．逆層クロマトグラフィーによりC2を精製し，質量スペクトログラフィーで既知のトリプシンインヒビターであるCMTI (Wieczorek et al., 1985)の混在がないことを確認した．この標品を用いてトリプシンインヒビター活性を測定したところ，約1.2 molのC2は10 μgのトリプシンを阻害することが明らかとなった (Fig. 8).

次に，C2ペプチドの機能発現がPV100のプロセシングに関係するか検討するため，精製したPV100のトリプシンインヒビター活性を測定した．遠心法により分離したPV100を用いて，トリプシンインヒビター活性を測定したところ，活性が認められなかった (Fig. 8).

VPEによりPV100がVicilinに変換される

PV100由来のペプチドはAsn-Glnの間で切断されていること (Fig.4, 7), PV100のN末端付近にも特異的なAsn-Gln (Gly)が見つかること (Fig. 3)よりVPEがPV100のプロセシングにも関与していると考えられた．そこで，ヒマ種子より精製したVPEを用いてPV100がプロセシングされる可能性を検討した (Fig. 9). カボチャの登熟子葉より単離したPAC小胞に含まれるタンパク質を基質としてVPEを作用させた．その結果，PV100は消失し，50 kDaに新しいペプチドがみられた．カボチャの乾燥種子のVicilinはアラニン残基の後ろより始まる (Fig. 2, 3)．そこで，VPEによって生じた成熟型VicilinのN末端を決定した．N末端はブロックされていたが，PV100よりプロセシングされた50 kDaのタンパク質はプ

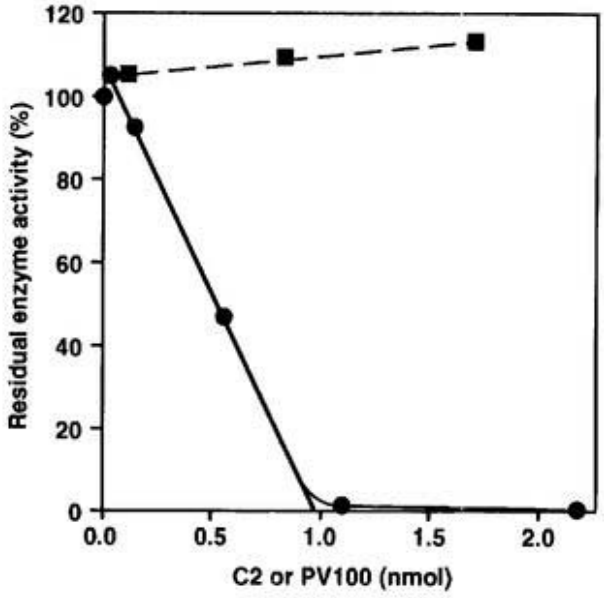


Fig. 8. PV100-derived C2 peptide functions as a trypsin inhibitor, but the precursor PV100 does not.

The C2 peptide was highly purified from soluble fraction of the protein-storage vacuoles of pumpkin seeds by HPLC. The PV100 was purified from the isolated PAC vesicles by a sucrose density gradient centrifugation . The reaction mixture contained 0 to 2.4 nmols of either the C2 peptide (closed circles) or the PV100 (closed squares), 10 μg of trypsin and 333 μg of BAPA in a 0.9-ml solution of 0.1 M Tris-HCl (pH 8.0) and 25 mM $CaCl_2$. The residual enzyme activity was monitored with absorbance at 405 nm.

ロテインボディにみられるVicilinのN末端配列より3アミノ酸残基上流のアスパラギン残基の次より始まっていることが明らかになった.

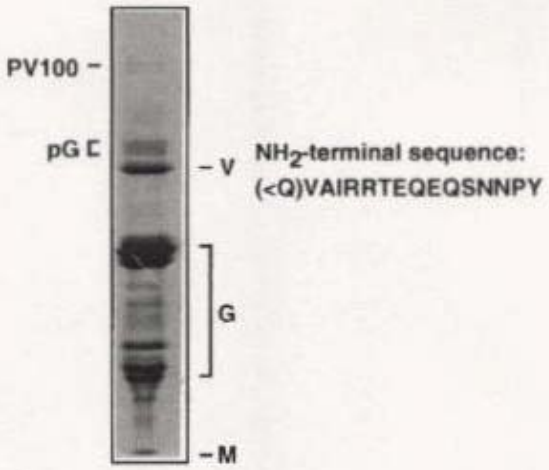


Fig. 9. *In vitro* processing of PV100 by purified VPE produces the vicilin-like protein.

PAC vesicles that contained PV100 were incubated with the purified VPE and then subjected to SDS-PAGE followed by staining with Coomassie blue. The resultant band corresponding to 50-kDa vicilin-like protein (V) that had been blotted to a GVHP membrane was incubated with pyroglutamate aminopeptidase and then was subjected to automatic Edman degradation. The determined N-terminal sequence corresponds to the sequence in PV100 as indicated by an arrow in Fig. 3. pG and G represent proglobulin and 11S globulin, respectively. The <10-kDa band (M) contained a mixture of the PV100-derived peptides and 2S albumin subunits that had been produced from pro2S albumin by VPE in the reaction.

考 察

PV100は種々の機能を持つ複数のタンパク質の前駆体である

液胞タンパク質は粗面小胞体で合成され，分泌輸送経路で液胞に蓄積していることが明らかになっている．通常，液胞タンパク質は小さな輸送小胞により液胞に送られてくる．しかし，種子の登熟期には，液胞のタンパク質が多量に蓄積してくるため，小胞体内腔に多量に蓄積し，蓄積したタンパク質はPAC小胞を経由して直接液胞に輸送される (Hara-Nishimura et al., 1998)．PAC小胞には，貯蔵タンパク質の前駆体の他に，輸送機構に必要なレセプターやGTP結合タンパク質が存在し，輸送のための小胞であることが確認できる (Shimada et al., 1994, 1997).

PV100は種子貯蔵タンパク質である11S globulin，2S albuminの前駆体の次に多いPAC小胞の構成成分である．このことから，PV100は輸送に関与するタンパク質というよりも輸送途中の液胞タンパク質の前駆体であると思われた．cDNAクローニングによりPV100は種子貯蔵タンパク質であるVicilinの前駆体であると同時に，プロテアーゼインヒビター活性を持つC2とREの前駆体でもあることが明らかになった．ナタマメ (*Canavalia ensiformis*)のCanavalin (Ng et al., 1992)やダイズ (*Glycine max*)のβ-conglycinin-β (Harada et al., 1989)にはシステイン領域がみられないが，ダイズのβ-conglycinin-α (Sebastiani et al., 1990)やアプランドワタ (*Gossypium hirsutum*)のα-globulin-A (Chlan et al., 1987)などはN末端側にプロセシングされるシステイン領域を持っているので，切り出されたペプチドはプロ領域として分解されるのではなく液胞に蓄積していることが考えられる．

液胞に蓄積するタンパク質が合成，輸送系の大部分を占める状況では，合

成，輸送系の簡略化が求められると思われる．多機能のタンパク質が一本のポリペプチドよりプロセシングされるPV100は，合成の簡略化の例かもしれない．ヒマ (*Ricinus communis*)の2S albuminや，タバコ (*Nicotiana alata*)のプロテアーゼインヒビターも一本のポリペプチドから同じ機能を持つ多数のタンパク質にプロセシングされる．ヒマの2S albuminは一つの前駆体から二つの成熟型タンパク質が生成する (Irwin et al., 1990)．また，タバコのプロテアーゼインヒビター前駆体は6つの活性型インヒビターにプロセシングされる(Atkinson et al., 1993).

PV100由来のペプチドの生理機能

PV100より4つのペプチド，C1，RE2，RE3，RE5が同定された．この中でC2はソバのトリプシンインヒビターと18%という低い相同性にも関わらず (Park et al., 1997)，トリプシンインヒビター活性が認められた．ソバより単離，精製されたトリプシンインヒビター(BWI-2b)は二つのCxxxCモチーフをおよそ5 kDaほどのタンパク質で，このCxxxCモチーフが向き合ってジスルフィド結合を形成し，ヘアピン構造をとっている．トリプシンに対する活性阻害部位であるアルギニン残基は二つのCxxxCモチーフの間にみられる．C2ペプチドのシステインはジスルフィド結合をしていること，CxxxCモチーフの間にアルギニン残基 (28番目， Fig. 7B)がみられることより，同様の構造をとって阻害していると思われる．ダイズのβ-conglycinin-α, アブランドワタのα-globulin-A, カカオ (*Theobroma cacao*)のVicilin (McHenry et al., 1992)，トウモロコシ (*Zar mays*)のGlobulin-1 (Belanger and Kriz, 1991)，ピーナッツ (*Arachis hypogaea*)のVicilin (Burks et al., 1995)のN末端側のシステイン領域はソバのトリプシンインヒビターよりも相同性が高いので，切り出されたペプチドがインヒビター活性を持っている可能性が考えられる．

種子に蓄積するC2は捕食動物から自分自身を守るために働いていると考えられる．カボチャ種子には強力なトリプシンインヒビター(CMTI)が蓄積しているが (Wieczorek et al., 1985)，C2ペプチドのアミノ酸配列の相同性はみられない．カボチャ種子はCMTIとC2ペプチドにより幅広く消化酵素を阻害すると考えられる．また，ソバのトリプシンインヒビターは抗原としての活性が高いことが報告されており (Park et al., 1997)，ピーナッツのアレルゲンとして同定されたタンパク質はシステインドメインを持ったVicilinであった (Burks et al., 1995)．C2ペプチドはトリプシンインヒビター活性だけでなく，捕食者にアレルギーを引き起し，それ以上食害を受けないために働いていることも考えられる．通常，防御機構に働くタンパク質は誘導性であるが，種子は乾燥しているので，素早いタンパク質の合成ができないため，常にインヒビター活性を持ったタンパク質が蓄積していると考えられる．

REペプチドもC2ペプチド同様に防御機構に働くと思われる．しかし，糸状菌やバクテリアに精製したこれらのペプチドを与えても菌の成長に変化はなかった．カボチャよりPBPを同定したNaisbitら(1988)は，このペプチドがマウス培養細胞に有毒であると報告しているが，精製過程で活性が減ることも報告している．よって，PBPは単独では働かず，他の因子と協調作用することにより抗菌活性を獲得すると思われる．

また，REペプチドは親水性アミノ酸に富んでいる．植物の種子の登熟後期には一群の親水性のLEA (Late embryogenesis abundant)タンパク質が蓄積する (Straub et al., 1994, Baker et al., 1988)．これらのタンパク質は乾燥ストレス時にも発現することが知られており，乾燥耐性を獲得するために必要であると考えられている．親水性REペプチドも同様に乾燥耐性を獲得するために必要であるとも思われる．

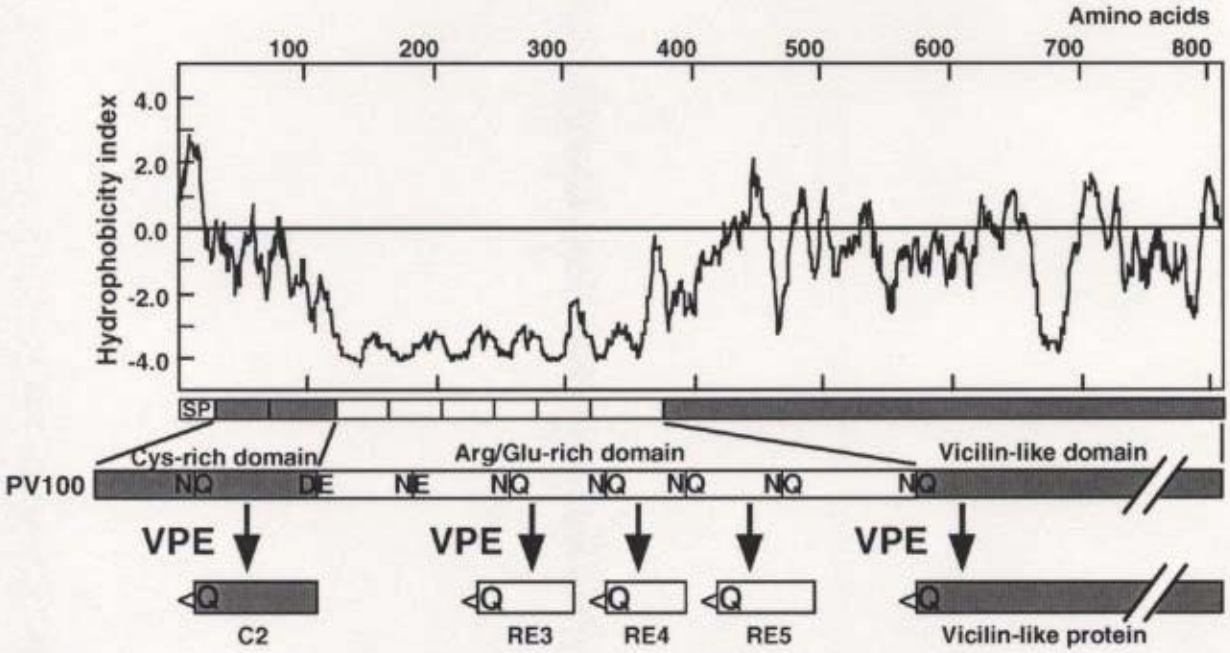


Fig. 10. Hydropathy profile of PV100 and a hypothetical mechanism for the VPE-mediated cleavage at Asn-Gln bonds to produce multiple seed proteins.

The mean hydrophobicity index was computed according to the algorithm of Kyte and Doolittle with a window of 10 residues. VPE is responsible for maturation of multiple seed proteins by cleaving Asn-Gln bonds that are found in the hydrophilic region of the PV100. Gln at the new NH_2 termini of the mature proteins might be spontaneously converted into pyroglutamate (<Q) under the acidic condition in the vacuoles. The cysteine-rich C2 peptide, the Arg/Glu-rich RE3, RE4 and RE5 peptides and the vicilin-like protein are produced. SP represents a signal peptide.

防御タンパク質としてのビシリンの機能

種子は動物にとっても重要な栄養源である．そのため，防御機構をになうPRタンパク質が種子に蓄積し種子自身を外敵から守っている (Huynh et al., 1992, Vigers et al., 1991)．また，種子貯蔵タンパク質自身がエネルギー源としての役割と種子自身を守る役割の両方をもっていることも考えられる．実際，ハツカダイコンの2S albuminには抗菌活性があり (Terras et al., 1992). サツマイモ *(Ipomoea batatas*)塊根の貯蔵タンパク質であるSporaminにトリプシンインヒビター活性があることが示されている (Yeh et al. 1997),

近年，ビシリンにも菌の細胞壁成分であるキチン結合活性があることが示されている (Sales et al., 1996)．ビシリンの抗菌活性は示されていないが，カボチャのVicilinがトリプシンインヒビターであるC2ペプチドや細胞毒性を持つREペプチドとともに合成されることから，貯蔵と防御機構の双方に働くタンパク質であることも示唆される．

PV100のVPEによるプロセシングと機能発現

これまでの研究により，VPEが11S globulinや2S albuminの前駆体のプロセシングに関与していることが明らかになっている．VPEはアスパラギンのC末端側を切断する酵素として単離された (Abe et al., 1993, Hara-Nishimura et al., 1993b)，PV100のプロ領域の繰り返し配列の境界にはVPEにより認識されると思われるアスパラギン残基がみられる．ハイドロパシーグラフよりこの領域は親水性であるため (Fig. 10)，VPEでプロセシングされる可能性が考えられた．実際に，ヒマより精製した酵素を用いて，PV100のプロセシングが確認され，*in vitro*プロセシングによる生じたVicilinはアスパラギン残基の後ろでプロセシングされていること，C2，RE3，RE4，RE5も同様にN末端はアスパラギン残基

の後ろで切断されていることより，VPEがこれらのペプチドへのプロセシングにも関与していると思われる．C末端はアスパラギン酸残基の後ろでプロセシングされている．カラスノエンドウよりとられたVPEはアスパラギン酸の後ろを切断することが明らかになっている (Becker et al., 1995)．また，当研究室でもヒマのVPEでアスパラギン酸残基の後ろを切断することが確認されている．このことより，C末端側のプロセシングもVPEが関与していると思われる．

成熟型のVicilin配列はアラニン残基の後ろから始まっている．カボチャ種子よりとられたアスパラギン酸プロテアーゼはプロテインボディに局在していることが示されており，オオムギ (*Hordeum vulgare*)種子からとられたアスパラギン酸プロテアーゼ同様に疎水性のアミノ酸の前後を切断している (Hiraiwa et al., 1997a, Sharkkinen et al., 1992)ので，アスパラギン酸プロテアーゼによりさらに切断されていると思われる．

トリプシンインヒビターの前駆体でもあるPV100には，インヒビター活性がみられず，プロセシング産物にのみ活性がみられること，REはC末端のプロセシングでその特徴である等電点のアルカリ側へのシフトが起こり，Naisbittら(1988)が報告しているPBPの特性と一致することは，これらのペプチドは液胞に運ばれ，プロセシングを受けて初めて機能や特性が生まれることを示している．C2もプロセシングにより活性が発現することから液胞で活性化することが必要であると考えられる．このように，機能発現にプロセシングが必要な低分子のペプチドとしてインゲンマメ (*Phaseolus vulgaris*)のα-アミラーゼインヒビターであるαAIが知られている．αAIは不活性のプロ型前駆体として合成され，活性の発現にはプロ領域の除去が必要である (Pueyo et al., 1993)．

今回，精製されたPV100由来のペプチドの内，同定されたのはC2とRE2, 3, 5のみであった．これらのタンパク質のN末端はグルタミン残基より始まっ

ているので，タンパク質のN末端がピログルタミル化する．N末端がピログルタミル化したタンパク質は，アミノペプチダーゼなどの消化酵素による分解が抑えられると考えられる．残りのC1，RE1，RE4は *in vivo* または精製途中で分解してしまった可能性が考えられる．また，C1にはVPEで認識されるようなアスパラギン残基がみられる．一つの可能性として，C1はVPEにより分解されることが考えられる．

第2章-分解型液胞における液胞プロセシング系の解析

シロイヌナズナには貯蔵組織型VPEとは異なる栄養組織型のVPE遺伝子が存在する．免疫電顕および細胞分画により，このVPE (γVPE)タンパク質が液胞に存在することを明らかにするとともに，γVPEがアスパラギン残基特異的なプロテアーゼ活性を持つことを示した．この結果より，γVPEは，栄養組織の分解型液胞内でプロセシング酵素として働いていると考えられる．分解型液胞におけるプロセシング系を解明するために，γVPEと同様の遺伝子発現パターンを示すシステインプロテアーゼであるRD21Aに注目し，RD21AがγVPEの基質特異性に合ったプロセシングを受けると考えられることから，これがγVPEの基質タンパク質となる可能性を検討した．

材料と方法

実験材料

実験材料としてシロイヌナズナ，コロンビア生態型 (*Arabidopsis thaliana* ecotype Columbia)を用いた．シロイヌナズナは，種子をバーミキュライト：パーライト1：1の割合に混合した土に播種し16時間光照射，8時間暗所，22℃で生育させた．

実験には無菌的にMS培地に生育させた植物体を用いた．0.5% Gellan Gum, 2.5 mM MES-KOHを含んだMS培地に，表面を2% antiformin，0.5% Triton X-100で滅菌したシロイヌナズナの種子を直径9 cmのシャーレに6粒蒔いた．種子の休眠を打破するために4日間，4℃でインキュベートした．22℃，連続光照射下

で3週間生育させた植物を用いて各種の処理を行った.

抗γVPE，抗RD21A抗体の作製

γVPEおよびRD21A特異的な抗体を大腸菌に発現させたタンパク質を用いて作製した．発現用のベクターにはpET32a (Novagen)を用いた．cDNA配列からオリゴヌクレオチド

γVPE-exp-F (5'-CCGGATCCAGAAGCGGTCCTGATGATGT-3'),

γVPE-exp-R (5'-CCCCCGGGTCTATGCACTGAATCCACGG-3'),

RD21A-F (5'-CCGGATCCTACGACGAGAAACATGGCGT-3'),

RD21A-W (5'-CCAAGCTTTTAGGCAATGTTCTTTCTGCC-3')

をデザインしDNA合成機 (Model 394, Applied Biosystems)を用いて化学合成し，プライマーとして用いた．ABRC より取り寄せたESTクローン (RD21A; 103I15T7，γVPE; 2H1T7)を鋳型として用い，PCRによりγVPEおよびRD21Aのコード領域を増幅し，制限酵素 *Hin* dIII/*Bam* HIで切断した後，同じように切断したpET32ベクターにタンパク質の読み枠が合うようにつなぎ，大腸菌BL21 (DE3)に導入した．誘導は，終濃度が2 mMになるようにIPTGを添加したLB培地で37℃，8-12時間培養することによって行った．大腸菌を50 mM sodium phosphate, pH 7.5, 50 mM imidazole, 0.3 M NaCl, 0.1 mM PMSFに懸濁し，超音波処理装置 (Model 450D, Branson)を使用し，Output 7，50% Cycleで氷上で5分間3回超音波処理し菌体を破砕し，10,000 x *g*で遠心した．この条件で，発現タンパク質は可溶化されなかったので，沈殿に0.1 M sodium phosphate, pH 7.5, 8 M ureaを加え超音波処理の後10,000 x *g*で遠心し粗抽出液を得た．粗抽出液をニッケルを結合させた HiTrap Chelatingカラム (Amersham Pharmacia Biotech)に通し0.1 M sodium phosphate, pH 7.5, 8 M ureaで洗浄した後，カラムに結合している発現タンパク

質を0.1 M sodium phosphate, pH 7.5, 8 M urea, 500 mM imidazolで溶出し，抗原として用いた．この段階で，γVPEは部分精製であったのでさらにSDS-PAGEによりタンパク質を分離し，ゲルより相当するペプチドを切り出し，PBSで平衡化した後抗原として用いた．

精製した抗原はFreund's adjuvantで，エマルジョン化させた後ウサギに皮下注射した．3週間後，4週間後に二度目，三度目の注射を行い，三度目の注射の1週間後に採血を行い血清を得た．

ウエスタンブロッティングとノザンブロッティング

SDS-PAGEとウエスタンブロッティングは一章に準じた．各処理をしたシロイヌナズナ葉よりタンパク質を 0.1% SDS, 10 mM Tris-HCl, pH 7.5で抽出し，21,000 x *g* で遠心した後，可溶性画分を試料とした．一次抗体は，抗γVPE抗体は1/100，抗RD21A抗体は1/1000の濃度で使用した．なお，抗γVPE抗血清は一度抗原で精製したものを使用した．

ノザンブロッティングは特に引用がない場合を除いて Sambrookら (1989) の方法に準じた．

各種処理をしたシロイヌナズナ葉より全RNAをISOGEN (Nippon Gene)により抽出した．抽出したRNAはLiClで一度精製した後に使用した．20 μgの全RNAを熱処理で変性させた後，formaldehydeを含む1% agaroseゲルにより分離した後，RNA量を確認するためにEtBr染色をした．電気泳動の後，RNAはナイロン膜 (Hybond N^+, Amersham Pharmacia Biotech)にキャピラリー法により転写した．

ABRC より取り寄せたESTクローン (RD21A; 103I15T7，γVPE; 2H1T7, SAG2; 111J23T7，Rubisco small subunit; 204L8T7，βtubulin; ATTS3206)より得た断片をプローブとして用いた．ハイブリダイゼーションの条件はKinoshitaら

(1995a, 1995b)の方法に従った.

免疫電子顕微鏡観察

試料の作製は一章に準じた．発芽後21日目のシロイヌナズナの本葉を5 mm角程度に細切し，固定液 (4 % paraformaldehyde, 1 % glutaraldehyde, 0.05 M sodium-cacodylate, pH 7.4, 0.06 M sucrose)に浸し，固定液が良く浸透するように軽く減圧浸透処理した後，室温で1時間固定した．試料はLR-White樹脂 (London Resin)に包埋し，超薄切片を作製しニッケルグリッドにマウントした．切片をブロッキング液 (1% BSA in PBS)で室温で1時間処理した後，ブロッキング液で1/500に希釈した抗γVPE抗体と4℃で一晩反応させた．PBS で洗浄後，ブロッキング液で1/20に希釈したプロテインA-金コロイド液 (Amersham Pharmacia Biotech)で室温で30分間処理した．その後，4 %酢酸ウランとクエン酸鉛で染色した．観察は透過型電子顕微鏡 (1200EX, JEOL)を使用して80 kVで行なった.

BY2細胞からのバキュオプラストの単離

BY2細胞より液胞を単離することは難しいと考えられたので，主に液胞を含み，核や他のオルガネラの混在が少ないプロトプラスト (バキュロプラスト)をSonobeら (1990, 1994)の方法で単離した.

新しい培地に植え継いだ後6日目のBY2細胞をミラクロス上に回収し，緩衝液P (25 mM Tris-MES, pH 5.5, 0.45 M mannitol)で洗浄した．細胞は酵素液 (1 % cellulase onozuka-RS (Yaklut), 0.1 % pectlyase (Seishin) in buffer P)で30℃，2時間処理し，細胞壁を分解した．遊離してきたプロトプラストはミラクロスで濾過し，濾液を700 x *g*で遠心した．沈殿は緩衝液V (25 mM Tris-MES, pH 7.0, 20 mM $MgCl_2$, 0.45 mM mannitol)に懸濁し，先程と同じ条件で遠心し，洗浄した．沈殿

を，30 % Percollを含む緩衝液Vに懸濁し，緩衝液を上層させ，10,000 x *g*で1時間遠心した．溶液をPercollの自然密度勾配により液胞を主に含む上層と，液胞が抜けたプロトプラスト(ミニプロトプラスト)からなる下層に分画した．

上層は10 %パーコールを含む緩衝液Vに懸濁し，緩衝液Vを上層させ，700 x *g*で遠心しバキュオプラストを緩衝液V中に回収した．下層は緩衝液Vで洗った．

VPEの酵母における発現

酵母 (*Saccharomyces cerevisiae*)におけるγVPEの発現には，親株としてYW5-1B (*MATa leu2 ura3-52 trp2*)由来の*pep4*株であるSTY1 (*MATa leu2 ura3-52 TRP2::pep4*)を用いた．また，発現用のベクターとして，酵母および大腸菌のシャトルベクターであるp415，p416を用いた．γVPEのcDNA配列からオリゴヌクレオチド

γVPE-ye-F (5'-CCGGATCCATGACACGTGTCTCCGTCGG-3'),

γVPE-exp-R (5'-CCCCCGGGTCTATGCACTGAATCCACGG-3'),

をデザインしDNA合成機 (Model 394, Applied Biosystems)を用いて化学合成しプライマーとして用いた．，ABRCより取り寄せたγVPEのESTクローン (2H1T7)のコード領域をPCRにより増幅し，p415の*Gal1*プロモータ下につなぎ，STY1に導入した．トリプトファン非要求性になった形質転換した酵母を選抜し，Hiraiwaら(1997b)の方法に従って培地の組成をガラクトースに変えることによりタンパク質を発現させた．タンパク質を発現した酵母は遠心により回収し，グラスビーズを用いて抽出用緩衝液 (50 mM sodium acetate, pH 5.5)とともに磨砕した後遠心し，上清をタンパク質試料とした．

VPE活性の測定

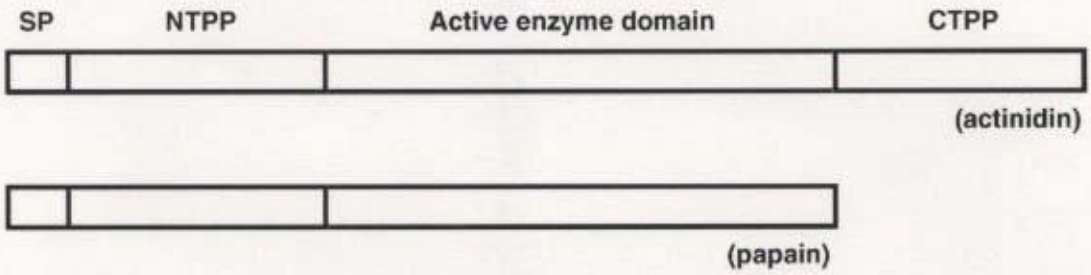
酵母に発現させたγVPEおよびBY2細胞のVPE活性をHara-Nishimura ら (1993b)の方法に従って測定した．カボチャの主要種子貯蔵タンパク質である 11S globulinのプロセシング部位を含むアミノ酸10残基 (SESENGLGGT)からなる合成ペプチド (NG10)を基質として用いた．20 mM sodium acetate, pH 5.5, 0.1 M DTT, 0.1 M EDTAからなるバッファー中に4.5 nmolの基質と抽出液を加え，37 ℃で3時間反応させた．VPEはNG10を二つのペプチド，P1; SESENとP2; GLGGTに分解する．残った基質と分解産物は，キャピラリー電気泳動 (30℃, 20 kV,10 mM sodium borate, pH 8.2)により分離し，200 nmの波長でペプチドを同定した．活性は，1分間に1 μmolのP2を精製する酵素量を1ユニットとして定義した．

結 果

栄養組織のVPEの基質タンパク質の検索

現在，栄養組織のVPEの基質として同定されているタンパク質は見つかっていない．そこで，まず栄養組織の液胞に存在すると考えられ，アスパラギン残基のC末端側で切断されるタンパク質を検索した．その結果，タバコ (*Nicotiana alata*)のトリプシンインヒビターであるNa-TI-II (Atkinson et al., 1993)，トマト (*Lycopersicon peruvianum*)のトリプシンインヒビターであるPI-I (Graham et al., 1985a)，タバコ (*Nicotiana tabacum*)の塩基性キチナーゼであるCBP20 (Ponstein et al., 1994)，パパイン型システインプロテアーゼに属するキウイ (*Actinidia chinesis*)のActinidin (Carne and Moore, 1978, Praekelt et al., 1988)が見つかった．シロイヌナズナにおけるこれらのタンパク質のホモログをデータベースより検索したところ，プロテアーゼインヒビターのホモログは見つからなかったが，Actinidinに相同性のあるタンパク質はゲノムシーケンス解析から分かっているものも含めて14存在していた．パパイン型のシステインプロテアーゼのほとんどはC末端側の延長配列を持たないが，Actinidin，イネ (*Oryza sativa*)のOryzain-αとOryzain-β (Watanabe et al., 1991)，トマト (*Lycopersicon esclentum*)のC14 (Schaffer et al., 1998)，エンドウマメ (*Pisum sativum*)のTPP (Granell et al., 1992)，ベイマツ (*Pseudotsuga menziesii*)のPseudotzain (Tranbarger and Misra, 1996)はC末端側に翻訳後に切断されると思われる延長配列を持っていた (Fig. 11A)．シロイヌナズナにもC末端側に延長配列を持つ乾燥誘導型のシステインプロテアーゼ (RD21A)があり (Koizumi et al., 1993)，延長配列の始まりにはアスパラギン残基がみられた (Fig. 11B)．そこで，RD21AのESTクローンをABRCから入手し栄養組織のVPEの基質である可能性を検討した．

A



B

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RD21A          GYLRMARNI-ASSSGKCGIAIEPSYPIKNGENPPNPGPSPPSPIKPPTQC   375
Oryzain-α      GYVRMERNI-KASSGKCGIAVEPSYPLKKGENPPNPGPTPPSPTPPPTVC   367
Pm Cys-P       GFIKLQRNLEGASTGMCGIAMEASYPVKKGANPPNPGPSPPSPVKPPTVC   371
Actinidin      GYMRILRNVGGAGT--CGIATMPSYPVKYNNQNHPKPYSSLINPPAFSMS   365
Papain         GYIRIKRGT-GNSYGVCGLYTSSFYPVKN*                       345
SAG12          GYMRIQKDV-KDKQGLCGLAMKASYPTI*                        346
SAG2           GYFKMEMGK-N----MCGIATCASYPVVA*                       358

RD21A          DSYYTCPESNTCCCLFEYGKYCFAWGCCPLEAATCCDDNYSCCPHEYPVC   425
Oryzain-α      DNYYTCPDSTTCCCIYEYGKYCYAWGCCPLEGATCCDDHYSCCPHEYPIC   417
Pm Cys-P       DNYYSCPESNTCCCMYDFGGYCYAWGCCPLNSATCCDDHYSCCPSDHPVC   421
Actinidin      KDGPVGVDDGQRYSA*                                     380

RD21A          DLDQGTCLLSKNSPFSVKALKRKPATPFWSQGRKNIA*               462
Oryzain-α      NVQQGTCLMAKDSPLAVKALKRTLAKPNLSFLFGNGKKSSA*           458
Pm Cys-P       DLDAQTCLKSRKDPFGTKMLKRTPAKPYWSLSG*                   454
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Fig. 11. RD21A is a cysteine proteinase of papain family and the precursor has a C-terminal propeptide .

(A) Two types of cysteine proteinases in papain family. All members of the family have an N-terminal propeptide (NTPP) to be cleaved post-translationally. Some members such as kiwi actinidin have a C-terminal propeptide (CTPP) the others such as papain do not have it.
(B) An alignment around the C-terminal regions of RD21A (GenBank accession No. D13043), oryzain-a (D90406), cysteine proteinase from douglas fir (*Pm* Cys-P, U41902), actinidin (X16466), papain (M15203), SAG12 (U37336) and SAG2 (PIR accession No. PQ0650). The identical amino acids to RD21A are shaded. A closed triangle indicates the cleavage site of the C-terminal propeptide of actinidin. Possible asparagine residues to be recognized by VPE are underlined. The amino acid numbers starting from the initiation methionine are indicated on the right.

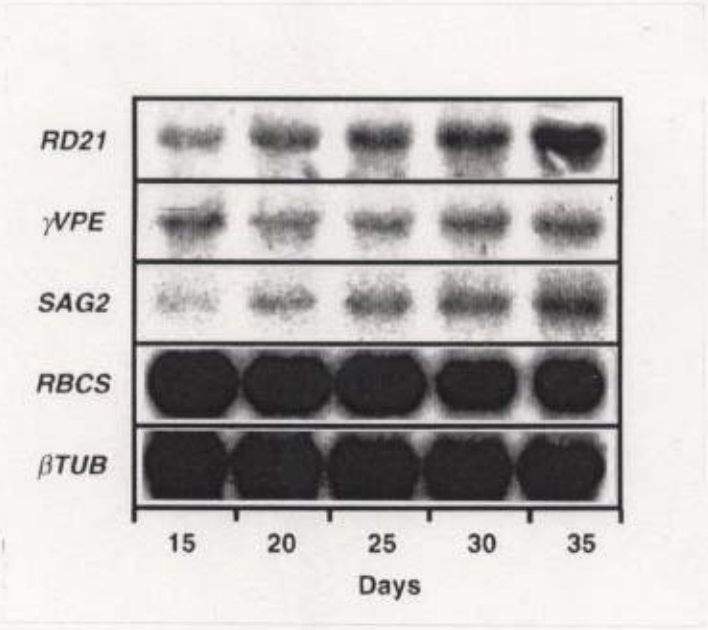


Fig. 12. Both *γVPE* and *RD21A* mRNAs are accumulated in the rosette leaves of *Arabidopsis* in association with senescence.

Total RNA was extracted from the primary and secondary rosette leaves of 15-, 20-, 25-, 30- and 35-day-old *Arabidopsis* plants. A Northern blot was performed with either probe *RD21A* or *γVPE*. It has been shown that senescence associated gene 2 (*SAG2*) is up-regulated and Rubisco small subunit gene (*RBCS*) is down-regulated during senescence. The probe of βtubulin (*βTUB*)was used as internal control on the blot.

また，γVPEはαVPEよりも発現量が多く，栄養組織において主要な働きをしていると考えられたので，γVPEを用いて実験を行った．

γVPEとRD21Aは葉の老化や乾燥ストレスで誘導されてくる

γVPEは老化の進行とともに誘導されてくる．そこで，まず両者の発現パターンが一致することを確認するためにRD21Aが老化に伴い誘導されるか検討した．発芽後，15から35日のシロイヌナズナの第1，2葉よりRNAを抽出し，ノザンブロッティングを行った (Fig. 12)．発芽後の時間経過とともに光合成系の示標酵素であるrbcSの発現量が減少すること，老化により誘導をうけるSAG2の発現量が上昇することより，葉の老化が順次進んでいることを示している．RD21Aの発現量は若い葉でも少し誘導されるが，老化とともに上昇しγVPEの発現と似たようなパターンを示した．

RD21Aの発現量はABA，NaCl，乾燥処理で増えてくることが明らかとなっている (Koizumi et al., 1993, Weaver et al., 1998)．そこで，γVPEの発現がRD21Aの発現に同調するか検討した．発芽後3週間目の植物体にABA，NaCl，乾燥処理を行い，未処理のものとγVPEの発現量を比較した (Fig. 13)．その結果，それぞれの処理においてγVPEが誘導されてくることが明らかとなった．このことより，γVPEとRD21Aの発現様式は似通っていることが示された．

γVPEとRD21Aの発現パターンがタンパク質レベルでも似ているかどうか検討した．大腸菌に発現させたγVPEとRD21Aタンパク質に対して作製した抗体を用いて種子および葉の抽出液に対してウエスタンブロッティングを行ったところ，それぞれ特異的なペプチドが確認された (Fig. 14A)．抗γVPE抗体はおよそ43 kDaのペプチド，抗RD21A抗体は33と43 kDaのペプチドを認識した．また，

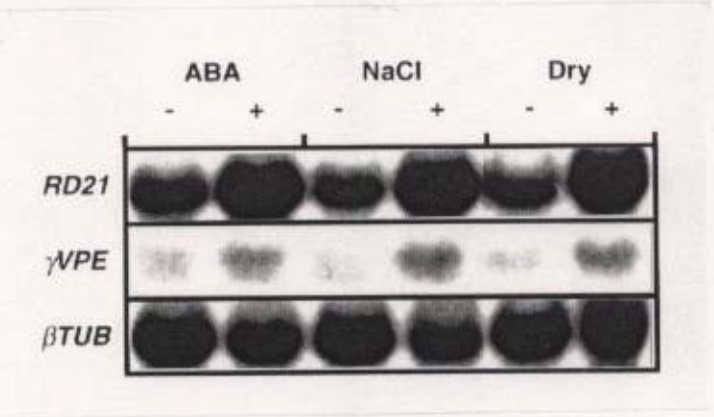


Fig. 13. Both *γVPE* and *RD21A* mRNAs are expressed in response to ABA, NaCl and dehydration.

Total RNA was extracted from the all aerial parts of *Arabidopsis* plants treated with ABA, NaCl and dehydration. A Northern blot was performed with either probe *RD21A* or *γVPE*. The probe of βtubulin (*βTUB*)was used as internal control on the blot.

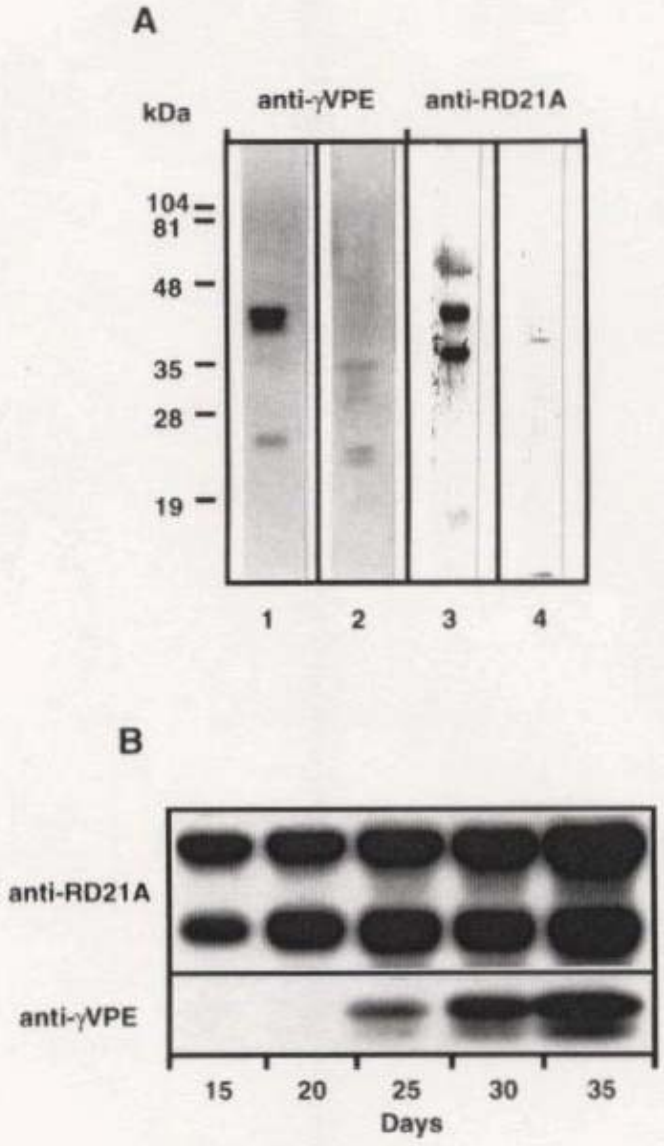


Fig. 14. The γVPE and RD21A proteins are specifically accumulated in vegetative tissues during senescence.

(A) Proteins were extracted from primary and secondary rosette leaves of 35-day-old *Arabidopsis* plants (lanes 1 and 3), and from the dry seeds (lanes 2 and 4). The extracts were subjected to SDS-PAGE, subsequently immunoblot with either anti-γVPE antibodies (lanes 1 and 2) or anti-RD21A antibodies (lanes 3 and 4). **(B)** Proteins were extracted from the primary and secondary rosette leaves of 15-, 20-, 25-, 30- and 35-day-old *Arabidopsis* plants. An immunoblot was performed with either anti-γVPE antibodies or anti-RD21A antibodies.

双方とも栄養組織のタンパク質のみを認識し，特にγVPEは貯蔵組織のタンパク質を認識しなかった．

この抗体を用いて，先程と同様に5から35日の第1，2葉よりタンパク質を抽出し，ウエスタンブロッティングを行った (Fig. 14B)．その結果，双方ともノザンブロッティングより得られた結果同様に，若い葉において少量のタンパク質の蓄積が認められ，その後葉の老化とともに増加し，タンパク質レベルでも発現パターンが一致していた．

γVPEは液胞に存在している

*in vivo*においてRD21AがγVPEの基質であるためには，両方の局在性が一致していなければならない．そこで，それぞれの抗体を用いてシロイヌナズナの葉に対して免疫電顕を行った (Fig. 15A, B)．その結果，抗γVPE抗体に反応する金粒子のシグナルが液胞のみに検出された．また，抗RD21A抗体に反応する金粒子のシグナルもγVPEと同様に液胞内にみられたが，免疫前の血清を用いたときにも液胞内に同程度のシグナルが検出された．このことより，γVPEは液胞内に存在することが確認されたが，RD21Aの細胞内局在性は確定できなかった．

さらに，液胞内にVPEが存在すること確認するためにBY2細胞より得たプロトプラストを液胞を含むバキュオプラスト画分と液胞を含まないミニプロトプラスト画分に分離し (Fig. 15C)，それぞれの画分よりタンパク質を抽出しVPEの活性を測定した．その結果，プロトプラストでは35.9 mU/mgタンパク質，バキュオプラストでは91.5 mU/mgタンパク質，ミニプロトプラストでは17.8 mU/mgタンパク質であった．液胞を含むバキュオプラストの画分にはVPEの活性がプロトプラストと比較して2.5倍に濃縮されていたが，液胞をほとんど含まないミニプロトプラストではVPEの活性がプロトプラストと比べておよそ半分

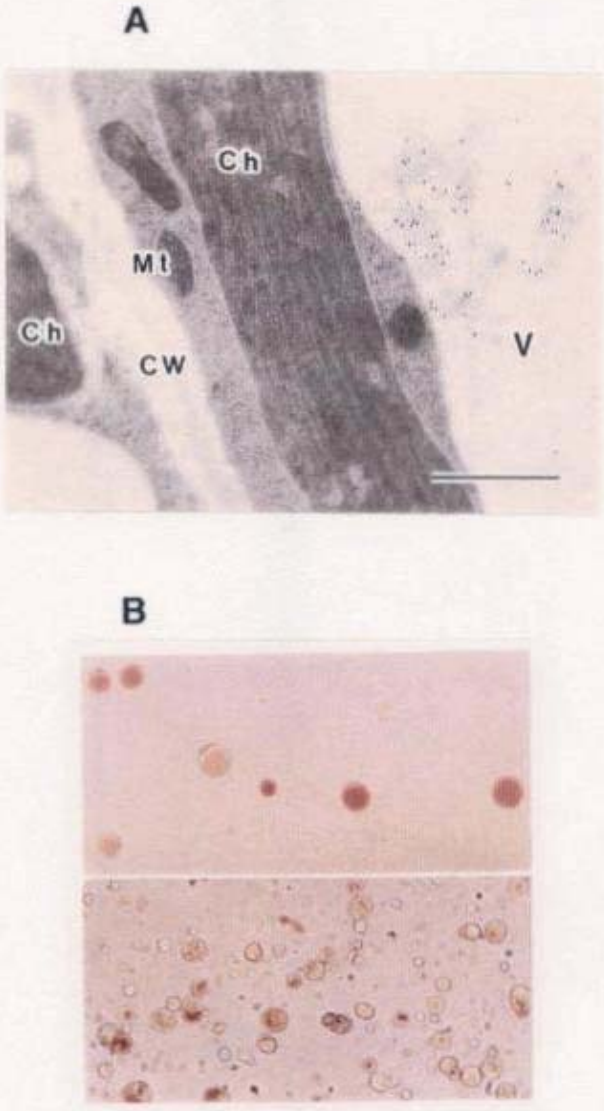


Fig. 15. The γVPE protein is localized in the vacuole of the vegetative tissues.

(A) An ultrathin section of one of the 5th leaves of a 21-day-old plant of *Arabidopsis* was incubated with anti-γVPE antibodies and then protein A-gold. Ch, chloroplast; CW, cell wall; Mt, mitochondrion; V, vacuole. Bar = 1 μm. **(B)** Vacuoplasts (upper) and miniprotoplasts (lower) were separated from the tobacco BY2 cells. These fractions were stained with neutral red and were subjected to phase-contrast microscopy.

に減っていた.

酵母に発現させたγVPEはアスパラギン特的な活性を示す

次に栄養組織のγVPEの性質を明らかにするため，γVPEを酵母の*pep4*株に発現させた．発現にはガラクトース誘導性のベクターを使用した．ガラクトース誘導後の酵母のタンパク質を抽出し，γVPEの発現量と活性を測定した (Fig. 16)．その結果，ウエスタンブロッティングよりガラクトース誘導後10時間目にプロ型前駆体の発現が確認されたが，VPE活性はみられなかった．その後，プロ型前駆体の量は減少し，代わりに成熟型と思われるおよそ43 kDaのペプチドがあらわれ，VPEの活性の上昇と一致した．ウエスタンブロッティングより，およそ5-10 kDaのところにガラクトース誘導後に上昇する小さなペプチドが蓄積し，その量は成熟型タンパク質と，前駆体タンパク質の発現量よりも明らかに多かった．γVPEの発現は，ヒマVPEの場合と比較して活性の増加が非常に遅かったが (Hiraiwa et al., 1997b)，これは発現したタンパク質が順次分解されるためであると思われる．

貯蔵組織のVPEであるヒマVPEを活性型のタンパク質として酵母に発現させると，Carboxypeptidase Yをプロセシングすることが知られている (Hiraiwa et al. 1997b)．そこで，抗CPY抗体を用いてウエスタンブロッティングを行ったところ，γVPEの活性の上昇とともにCPYの成熟型への変換がみられた．このことより，栄養組織のγVPEは種子のVPE同様に酵母のProteinase Aの代わりとなることが示された．また，*in vitro*でのRD21Aの成熟型への変換を観察するために，RD21Aを酵母内に発現させることを試みたが，発現させることができなかった.

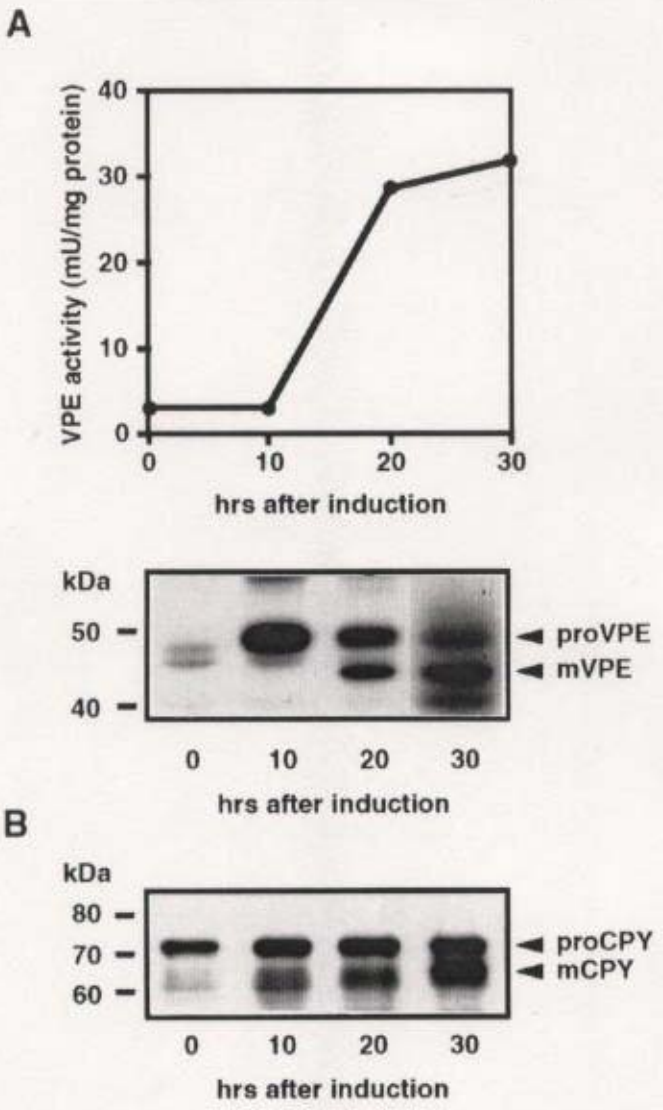


Fig. 16. The inactive γVPE precursor is self-catalytically converted into the mature active enzyme in the transformant cells of *pep4* strain of *Saccharomyces cerevisiae*.

(A) The γVPE gene flanked with the *GAL1* promoter was introduced into the yeast *pep4* strain. Total proteins from the transformant cells were subjected to SDS-PAGE and then to immunoblot with anti-γVPE antibodies. The γVPE precursor (proVPE) was converted into the mature γVPE (mVPE) during incubation with galactose.
(B) Immunoblot of the same extracts as above was performed with anti-carboxypeptidase Y (CPY) antibodies. The active VPE expressed in the *pep4* transformant was responsible for the conversion of proCPY into mCPY, instead of *PEP4* gene product, proteinase A.

考 察

栄養組織におけるVPEの役割

種子におけるVPEを中心とした貯蔵タンパク質のプロセシングのメカニズムは第一章で述べた．栄養組織にも，VPEホモログが存在するので，液胞内プロセシング系が存在すると思われるが，その実体は不明である．シロイヌナズナを使用した免疫電子顕微鏡観察と，BY-2を使用した細胞分画の結果より，液胞内に栄養組織特異的なVPEが存在し，かつ，VPE特異的なアスパラギン残基のC末端側を切断する活性が見られた (Fig. 15)，さらに，酵母にγVPEを発現させた場合，VPE活性の上昇とともに，酵母のCPYの成熟型への変換が見られた (Fig. 16)．このことは栄養組織のVPEも貯蔵組織のVPE同様にアスパラギン残基特異的な活性を持ち，液胞内のプロセシング酵素として働くことを示している．シロイヌナズナにおいて，栄養組織のVPEはαVPEとγVPEの二種類が単離され，その発現量はαVPEよりもγVPEの方が多い．また，γVPEは老化の初期に発現量が上昇するが，αVPEは老化の後期に発現する．よって，栄養組織では，γVPEがプロセシング酵素として主要な役割を果たしていること，αVPEはγVPEと異なる役割を果たしていることが示唆される．

酵母の*pep4*株に発現させたγVPEは分子量の大きなプロ型として発現し，時間の経過とともに分子量の小さな成熟型に変換し，VPE活性が上昇した (Fig. 16)．このことは，γVPEは不活性型で合成され，液胞に到達した後に自己活性化する事を示している．貯蔵組織においてイニシャルなプロセシングを行うヒマのVPEも液胞内で自己活性化する (Hiraiwa et al., 1997b)．また，酵母の液胞におけるプロセシング酵素であるProteinase A，動物のリソソームにおけるプロセシング酵素であるCathepsin Dは自己活性化することが報告されている (van den

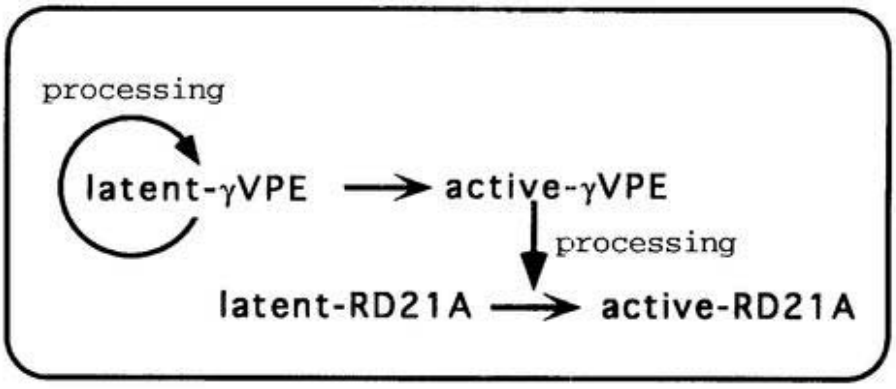


Fig. 17. A schematic model for activation of RD21A by a post-translational removal of the C-terminal propeptide by the VPE.

The γVPE precursor is synthesized as a latent form on the endoplasmic reticulum in the cells and then transported to the vacuoles. The inactive precursor is converted into the mature active γVPE after arrival at the vacuoles. The active γVPE might be involved in the maturation and activation of vacuolar proteins such as RD21A.

Hazel et al., 1992, Conner and Richo, 1992). よって，γVPEは液胞プロセシング系の中心となり，自己活性化した後，RD21Aなど他の液胞タンパク質を順次活性化していくと考えられる (Fig. 17).

RD21Aタンパク質の発現量が老化時に増加し，乾燥，塩ストレス，アブシジン酸処理によりγVPEのmRNAの発現量が増加することが示された．このことは，γVPEの発現が基質となるRD21Aの発現量の増加に応じて増えることを示している．しかし，傷害処理ではγVPEの発現が誘導されるのに対してRD21Aの発現はみられなかったので，γVPEがRD21Aだけでなく他のタンパク質のプロセシングにも関与していることを示唆している (第3章).

RD21Aは栄養組織におけるVPEの基質と考えられる

カボチャ種子では*in vitro* で貯蔵タンパク質である2S albumin, 11S globulin, Vicilinがアスパラギン残基を特異的に認識するVPEにより成熟型に変換される．栄養組織ではタバコのNa-TI-II, トマトのPI-I, タバコのCBP20, キウイのActinidinがアスパラギン残基のC末端側でプロセシングされることが明らかとなっているので，VPEの基質分子と考えられた．しかし，シロイヌナズナにはタバコやトマトに見られるプロテアーゼインヒビターは存在せず，塩基性キチナーゼのC末端のアスパラギン残基は保存されていないので (Samac et al., 1990), シロイヌナズナのVPEはこれらのタンパク質のプロセシングには関与していないと思われる．

シロイヌナズナのActinidinホモログであるRD21Aのプロ型前駆体の推定分子量はおよそ48 kDaであるが，ウエスタンブロッティングよりRD21Aは33, 43 kDaのタンパク質であることが示され，RD21Aがプロセシングされていることがわかった．シロイヌナズナのRD21AはActinidinと同じく，C末端側の延長配列

を持ち，プロセシング部位と思われる付近にはアスパラギン残基が存在する．また，RD21Aはシグナルペプチドを持つので，液胞に蓄積すると思われる．これらのことよりRD21Aの成熟型へのプロセシングにはγVPEが関与していると考えられる．結晶構造解析より成熟型ActinidinのC末端は分子表面に露出していることが示されたが (Baker, 1980)，RD21Aもこのような構造を取り，VPEがアクセスしやすくなっていると思われる．

動物のパパイン型システインプロテアーゼのホモログであるCathepsin B，LのN末端は自己阻害領域として働いており，プロ型前駆体には活性がみられない (Carmona et al., 1996, Fox et al., 1992)．Actinidinも不活性な前駆体として合成されるが，N末端延長配列が付加したままのActinidinを人工的にデザインし，タバコ培養細胞に発現させてもプロテアーゼ活性を持っていたので (Paul et al., 1995)，ActinidinのC末端プロペプチドはプロテアーゼ活性を抑制する働きがあると考えられる．RD21AのC末端領域はOryzain-αとOryzain-β，C14，TPP，Pseudotzainにもみられ，良く保存されているが，Actinidinとは相同性が低いので (Fig. 11)，RD21AのC末端がプロテアーゼ活性の制御に関わっているかは不明である．

この領域は，システインに富みホモログ間で配列が保存されている．このことより，システインがジスルフィド結合をとって，特徴的な構造をとっていることが考えられる．このシステインに富む領域に相同性を示すタンパク質を検索したところ，ヒトやマウスの成長因子の一つであるグラニューリンが得られた (Bhandari et al., 1992, Bateman and Benett, 1998)．グラニューリンは，およそ6 kDaの小さなペプチドである．このペプチドの受容体は単離されていないが，システインに富み，特徴的な構造をとっている．このことよりRD21A由来のC末端ペプチドはプロセシング後，分解されずに液胞内に蓄積し，何らかの機能

を果たしていることが考えられた.

分解型液胞における多段階の液胞プロセシング系

RD21Aと同じように老化で誘導されてくるパパイン型システインプロテアーゼであるSAG2やSAG12にはC末端延長配列がみられないため，C末端のプロセシングは起こらないと思われる (Fig. 11) (Hensel et al. 1993, Lohman et al. 1994). また，RD21A，SAG2やSAG12のN末端の延長配列にはアスパラギンやアスパラギン残基が見つかるので，VPEによるプロセシングを受けると思われる．しかし，これらのプロテアーゼのN末端プロセシング部位付近はよく保存されており，疎水性アミノ酸のロイシン残基がみられるので，最終的なN末端のプロセシングにはアスパラギン酸プロテアーゼが関与している可能性が考えられる．ケツルアズキ (*Vigna mungo*)のシステインプロテアーゼであるSH-EPは多段階にプロセシングされる (Yamauch et al., 1992)．また，酵母のCPYも多段階プロセシングを受け活性化する (Mechler et al., 1987, Hirsch et al., 1992)．栄養組織の液胞は，プロテアーゼに富んだオルガネラであるので，多段階のプロセシング系が存在すると考えられる.

第3章-栄養組織における液胞プロセシング酵素 (Vacuolar processing enzyme)の発現調節機構

栄養組織型γVPE遺伝子が傷害により全身的に発現誘導されることを明らかにした．本章ではこの結果を基に，γVPEの遺伝子発現調節の機構をさらに詳しく解析した．植物が傷害を受けると種々の遺伝子が活性化されることや，傷害による遺伝子発現にはジャスモン酸やエチレンが深く関わっていることが知られている．しかし，傷害によるγVPE遺伝子の発現にはジャスモン酸やエチレンの関与があまりみられず，他の傷害誘導性の遺伝子とは発現パターンが異なった．このことは傷害による全身的な遺伝子の活性化には複数の経路があることを示している．また，傷害によるγVPEの発現には新たにアブシジン酸が関与することも分かってきた．以上の結果は，傷害を受けた栄養組織の分解型液胞内に液胞プロセシング系が発現されてくることを示している．

材 料 と 方 法

実験材料

実験材料として用いたシロイヌナズナ，コロンビア生態型 (*Arabidopsis thaliana* ecotype Columbia)の扱いは，第2章に準じて行っている．

傷害および各種ホルモン処理には無菌的にMS培地に生育させた植物体を用いた．0.5% Gellan Gum，2.5 mM MES-KOHを含んだMS培地に，表面を2% antiformin，0.5% Triton X-100で滅菌したシロイヌナズナの種子を直径9 cmのシャーレに6粒蒔いた．種子の休眠を打破するために4日間，4℃で処理した後，

22℃，連続光照射下で生育させた．22℃に移した後3週間後の植物を用いて各種の処理を行った．

傷害およびホルモン処理

傷害は鉗子で葉を横に挟むことにより2箇所に与えた．エチレン処理は最終濃度が10 ppmになるように1.5 Lの容器に加え，シロイヌナズナを生育させているシャーレごと入れ密閉した．ジャスモン酸は最終濃度が40 μMになるようにmethyl jasmonate を与えた．アブシジン酸は最終濃度が2.5 Mになるように与えた．また，エチレン受容体の競合阻害剤として 2,5-norbornadieneを終濃度が150 μl/Lになるように1.5 Lの容器に加え，シロイヌナズナを生育させているシャーレごと入れ密閉した．

ノザンブロッティング

ノザンブロッティングの方法は Sambrookら (1989)に準じて行った．各種処理をしたシロイヌナズナ葉より全RNAをISOGENにより抽出し，1%アガロースゲル電気泳動の後，RNAをナイロン膜 (Hybond N^+, Amersham Pharmacia Biotech)にキャピラリー法により転写した．

ABRCより取り寄せたEST clone (γVPE; 2H1T7，27-kDa Atvsp; ATTS1295 (Berger et al., 1995)，GST1; 206N21T7，GST5; 44G9T7，soybean peroxidase (prxCb) homolog; B25T7 (Jabs et al., 1996)，rbcS; 204L8T7，βtubulin; ATTS3206)より得た断片をプローブとして用いた．ハイブリダイゼーションの条件はKinoshitaら(1995a, 1995b)の方法に従った．

GUSコンストラクトの作製

プロモータGUSの形質転換体を作製するために, γVPEのプロモータ領域 (-2036から+11)に対するプライマー

gVPE-prom-F (5'-CCAAGCTTCCGGTTTCATTATGGTTAAG-3'),

gVPE-prom-R (5'-AACCCGGGAGACGTGTCATCGTTGTGG-3')

をゲノムシーケンスを基に作製し，ゲノムクローンを鋳型としてPCRにより増幅した．増幅したDNAをクローニングベクター (pBluescript)に導入し，塩基配列を確認した後，制限酵素*Hin* dIII/*Sma* Iで切り出した後，バイナリーベクター (pBI121HmRV)にβ-glucuronidaseに読み枠が合うようにつないだ．作製したプラスミドはアグロバクテリアEHA101に導入した後，鉢植え7週間めぐらいのシロイヌナズナ植物体にバキューム法により感染させた．感染させたシロイヌナズナは3週間後にT1種子を採取し，100 mg/l Kanamycin，50 mg/l Hygromycin，0.5% Gellan Gum，2.5 mM MES-KOH (pH 5.7)を含んだMS選択培地に播種し，生えてきたものを鉢に移植した．鉢植えしたT1植物体は自家受粉させT2種子を得た．得られたT2種子を実験に用いた．

GUS染色

T2種子を0.5% Gellan Gum，2.5 mM MES-KOHを含んだMS培地に無菌的に3週間連続光照射下，22℃で育てた．植物体の第5，6葉にピンセットで傷を付け，2日後にサンプリングした．サンプリングした葉は傷を付けないようにエッペンドルフチューブに入れ, およそ500 µl の染色液 (0.5 mg/ml X-gluc, 0.1% (W/V) TritonX-100，100 mM sodium phosphate, pH 7.4)を加えた．サンプルは染色液が良く浸透するように軽く減圧浸透処理した後に37℃で一晩インキュベートした．その後，エタノールシリーズで組織よりクロロフィルを脱色し，かつ固定を行った．T2種子は遺伝的にヘテロな集団であるので，形質転換した植物体

と野生型の植物体が含まれている．γVPEは排水組織に発現することがわかっているので，排水組織が染色されるものを形質転換体としてさらに観察した．

結 果

VPEは傷害により誘導されてくる

γVPEの発現は傷害により上昇してくる．そこで，傷害によるγVPEの発現を時間を詳しく追って調べた．発芽後3週間の植物体の第5，7葉に鉗子で2箇所傷を付け一定時間後に第5-8葉をサンプリングし，ノザンブロットを行った (Fig. 18)．その結果，傷害後12時間後から急速な発現の誘導がみられ，72時間後まで上昇し続けた．この発現の上昇は傷を付けた葉のみではなく，傷を付けなかった葉にもみられた．そこで，種々のストレスにより誘導される遺伝子がどのように発現してくるかを観察した．その結果，傷害により発現するVSP，GST1，GST5，prxCbはγVPEよりも早く誘導された．また，これらの遺伝子の内のほとんどが傷を受けた葉のみならず，傷を受けなかった葉においても発現の上昇がみられたが，prxCbは傷害を受けた部位のみに発現の上昇がみられた．光合成に必要な遺伝子である，RBCSの発現は傷を付けた部位でのみ一時的に減少し，老化で誘導されるRD21Aの発現誘導は起こらなかった．このことから，傷害によるγVPEの発現誘導は傷を受けた部位と受けなかった部位両方にみられ，その発現は種々の遺伝子の発現よりも遅いことが明らかになった．

傷害におけるγVPEの誘導はジャスモン酸，エチレン非依存的である

傷害による全身的な遺伝子発現の誘導に関わる物質としてジャスモン酸，エチレンがもっとも良く知られている (Zarembinski and Theologis, 1994, Creelman and Mullet, 1997)．そこで，一般的によく調べられているエチレンとジャスモン酸がγVPEの発現に関与しているかを検討した．無菌的に育てたシロイヌナズナ

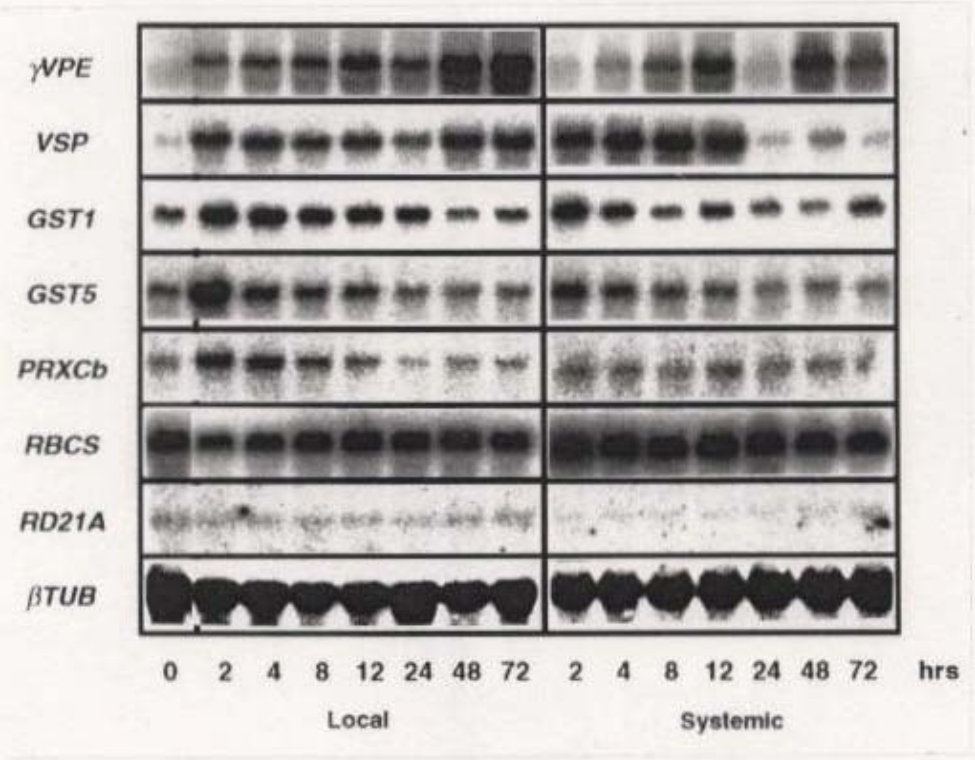


Fig. 18. The pattern of the wound-inducible gene expression of *γVPE* is different from those of the known wound-responsive genes.

Total RNAs were extracted from the local or systemic 5 to 8th leaves of 3-week-old *Arabidopsis* plants at the interval after wounding. A Northern blot was performed with each probe of *γVPE*, 27-kDa vegetative storage protein of *Arabidopsis* (*VSP*), glutathion *S*-transferases (*GST1* and *GST5*), peroxidase Cb (*PRXCb*), *RD21A* and Rubisco small subunit (*RBCS*). The *γVPE* mRNA was accumulated in response to wound-treatment in the local and systemic regions of the plants. The probe of βtubulin (*βTUB*)was used as internal control on the blot.

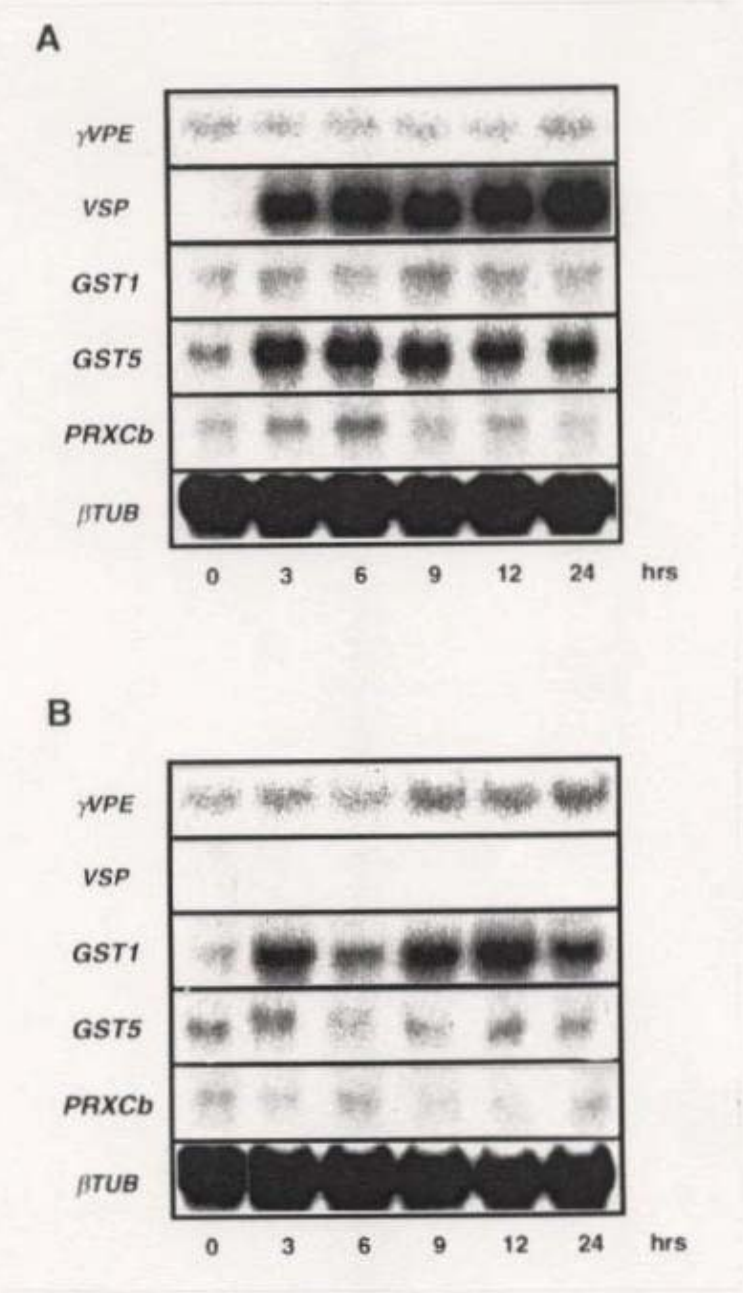


Fig. 19. Deferential responses of wound-inducible genes in response to exogenous plant hormones.

Total RNA was extracted from the 5 to 8th leaves of 3-week-old *Arabidopsis* plants that had been treated with either methyl jasmonate (**A**, final concentration of 40 μM) or ethylene (**B**, final concentration of 10 ppm). Probes used for Northern bolt were the same as shown in Fig. 18.

にジャスモン酸およびエチレンを与え，γVPEの発現が上昇するか検討した (Fig. 19)．その結果，γVPEの発現量はエチレン添加後9時間後に上昇したが，ジャスモン酸は添加後24時間後に少し上昇したのみだった．VSP，GST5はジャスモン酸のみ，GST5はエチレンのみに反応したが，添加後3時間後にすでに発現量が上昇していた．prxCbはジャスモン酸およびエチレン添加6時間後にわずかに反応した．このことより，ジャスモン酸，エチレン添加におけるγVPEの発現は他の傷害を受け全身的に発現する遺伝子よりも低く，遅いことが明らかとなり，これらのホルモンが傷害におけるγVPEの発現誘導に関わる可能性が低くなった．そこで，さらにこのことを確認するためにエチレンあるいはジャスモン酸のシグナル伝達がみられなくなった植物体での傷害におけるγVPEの反応を観察した (Fig. 20)．トマトや，シロイヌナズナにおいて，エチレンの受容の阻害剤であるノルボルナジエンを作用させることにより，傷害の反応がみられなくなることが知られている(Zarembinski and Theologis, 1994, Bleecker and Shaller, 1996)．そこで，ノルボルナジエンを与えたシロイヌナズナに鉗子で傷を付けたところ，γVPEの発現の抑制はみられなかった．また，ジャスモン酸に反応がみられない突然変異体，*jar1-1* (Staswick et al., 1992)に対して傷害を与えたところ，同様にγVPEの発現の抑制はみられなかった．

VPEの誘導はアブシジン酸が関与している

傷害による全身的な遺伝子発現にはシグナルとしてジャスモン酸，エチレンの他にアブシジン酸が関与していることが知られている．そこで，アブシジン酸の関与を調べるため，発芽後3週間の植物体にアブシジン酸を与え，γVPEの発現を観察した (Fig. 21)．その結果，γVPEの発現量はアブシジン酸添加後24時間後急激に増加した．

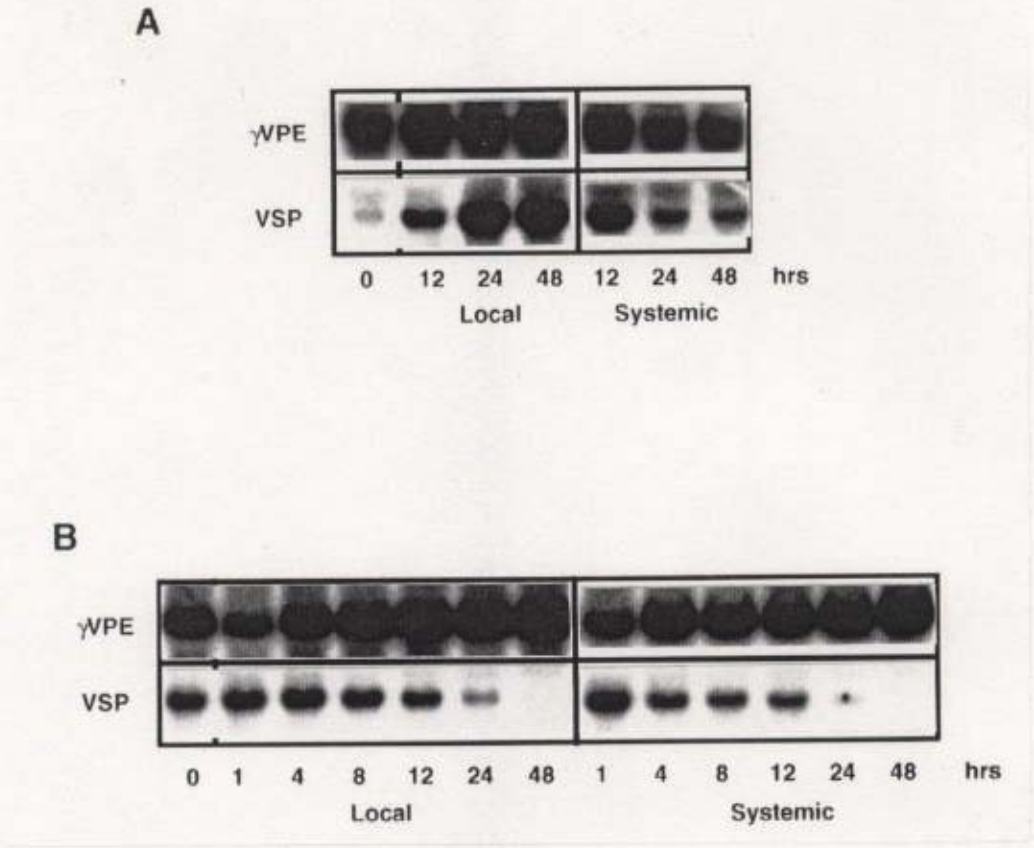


Fig. 20. Wound-responsive expression of the *γVPE* gene is independent from induction with jasmonate or ethylene.
(A) Total RNA was extracted from 5 to 8th leaves of 3-week-old plants of jasmonate-insensitive *Arabidopsis* mutant *jar1-1*. A Northern blot was performed with either probe *γVPE* or *VSP*.
(B) Total RNA was extracted from 5 to 8th leaves of 3-week-old *Arabidopsis* plants treated with 2,5-norbornadiene, ethylene perception inhibitor. A Northern blot was performed as descried in materials and methods.

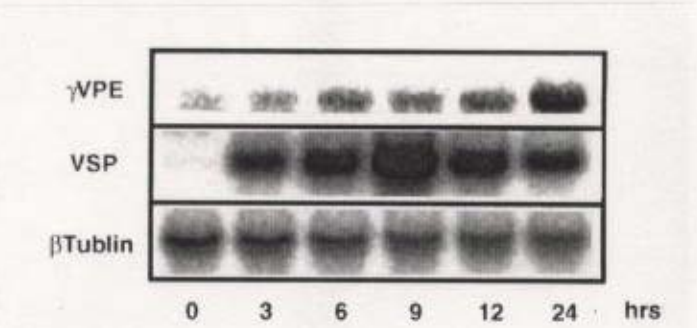


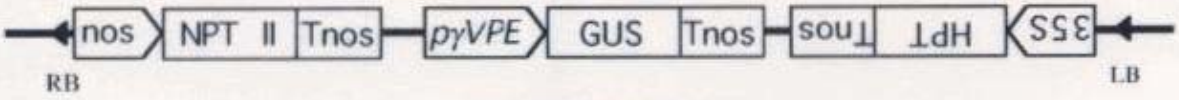
Fig. 21. The *γVPE* mRNA is expressed in response to abscisic acid (ABA).

Total RNA was extracted from 5 to 8th leaves of 3-week-old *Arabidopsis* plants treated with 2.5 mM abscisic acid (ABA). A Northern blot was performed as descried in materials and methods.

傷害を受けた維管束の周りにγVPEの発現はみられる

γVPEの発現を組織レベルで観察するために，γVPEの発現をプロモーターGUSの形質転換体を使用し，GUSの組織染色を行った (Fig. 22)．その結果，傷を付けない場合でも維管束は良く染まるが，傷を受けた部位に強い反応が現れた．また，切断された維管束の上流はさらに強く染色され，離れた部位ではそれほど強く染まらなかった．

A



pBI121HmRV

B

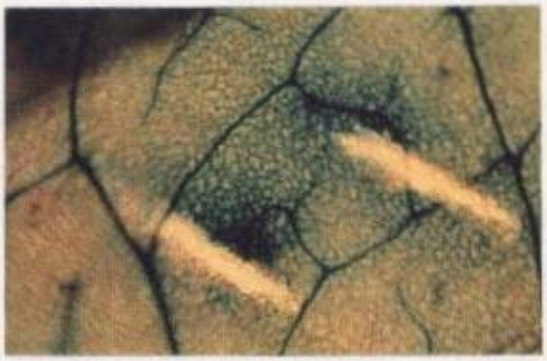


Fig. 22. The γVPE mRNA is expressed mainly the veins around the wounding sites.

(A) A construct of *γVPE* promoter-*GUS* gene (*pγVPE::gus* gene) that was introduced into Arabidopsis plants. nos, nopaline synthase promoter; NPT, neomycin phosphotransferase; Tnos, nopaline synthase terminator; *pγVPE*, *γVPE* promoter, GUS, β-glucuronidase; HPT, hygromycine phosphotransferase; 35S, cauliflower 35S promoter.
(B) Histochemical localization of GUS activity was found on the veins around the wounding sites of the transgenic plants with the *pγVPE::gus* The plant that had been incubated for 2 days after wound treatment was used for the GUS staining.

考 察

γVPEは傷害により全身的に誘導される

第二章で，γVPEが老化と共に誘導されてくることが示され，本章では，傷害によりγVPEが誘導されることが明らかとなった．しかし，傷を受けた葉のrbcSの発現量は減少するが，一時的であること，老化により誘導されてくる遺伝子であるRD21A，SAG2の発現量には変化がないこと (Fig 18)より，傷害による遺伝子の誘導は，明らかに老化による誘導とは異なっている．植物が傷を受けたときに全身的に誘導される遺伝子のシグナルとして同定されているものとして，一番良く知られているのがエチレンとジャスモン酸である．植物が傷害を受けると，ジャスモン酸やエチレン合成系の酵素が活性化され，それにより合成されたジャスモン酸とエチレンはシグナルの伝達物質として傷を受けていない部位に到達して防御遺伝子の発現をうながす．しかし，γVPEの発現はジャスモン酸の添加により誘導されなかったこと，またエチレンで誘導されてもその量は少ないことより，傷害によるγVPEの発現の直接的な誘導には関わっていないのではないかと考えられた．近年の研究で，傷害誘導性の遺伝子の発現にはエチレンとジャスモン酸が協調して働くこと必要であることが明らかにされている (O'Donnell et al., 1996)．予備的な実験ではあるがエチレンとジャスモン酸を同時にシロイヌナズナに与えた時は，RBCSの発現が押さえられること，老化で誘導されるSAG2やRD21Aの発現が誘導されること，葉の色が薄くなることより老化のような現象を引き起こしていると考えられた．外からホルモンを与えることによる傷害の模擬的な反応は，傷害における反応を厳密に反映していないためこのような結果が得られたと思われた．

また，シロイヌナズナのαVPE，γVPEは全身獲得性抵抗反応のシグナルの

一つであるサリチル酸に反応することが示されている．非親和性の病原菌が植物に感染したときに，植物体が抵抗性遺伝子を介して過敏感抵抗反応をおこし，サリチル酸の合成が起こる．サリチル酸はジャスモン酸の合成およびジャスモン酸の反応を阻害することが知られている(Pena-Cortes et al.,1993, Doares et al.,1995)．しかしながらタバコにおいて傷害誘導性タンパク質であるプロテアーゼインヒビターは菌感染時にも全身的に合成されることを考えるとサリチル酸の働きは単にジャスモン酸のシグナル伝達を押さえるだけではないと考えられる．病原菌誘導性のタンパク質を常に発現するシロイヌナズナの*cpr* (Constitutive express PR-proteins)突然変異体の中には，傷害誘導性のタンパク質を常に発現しているものがあるので(Clarke et al., 1998)，これらの突然変異体のVPEの発現パターンを解析することにより，傷害によるVPEの発現メカニズムを調べることができると思われる．

傷害で誘導されてくるγVPEの基質タンパク質

前章で，老化組織ではRD21AがγVPEの基質となりうる可能性が示唆された．しかしながら，RD21Aは傷害では誘導されなかった．また，傷害で誘導されてくるVSPは液胞に蓄積するが，アスパラギン残基でプロセシングされるような部位は見つからなかった．これらのことより，傷害におけるγVPEの基質分子は不明である．傷害誘導性のThioninは細胞外に放出される抗菌性タンパク質として同定されているが，オオムギ(*Hordeum vulgare*)においてThioninの液胞型のホモログが存在していることが示されている(Reimann-Philipp et al., 1989)．また，同じオオムギよりとられているThioninホモログはアスパラギンの後ろでプロセシングされることが示されているが (Romero et al., 1997)，多くの植物のThioninではアスパラギン残基は保存されておらず，傷害後速やかに誘導される

ため (Bohlmann, 1994)，VPEの基質とは考えにくい．タバコでは，前出のプロテアーゼインヒビター (NaTI-II)が液胞に蓄積し，アスパラギン残基の後ろでプロセシングされ活性を発現することが知られている．また，菌感染で誘導されるタバコの塩基性キチナーゼ (CBP20)はC末端側に液胞への輸送シグナルを持ったプロペプチドがある．このプロペプチドは液胞に到達した後にアスパラギンで切断される．タバコの培養細胞であるBY2にもVPE活性がみられることより (第2章)γVPEとホモロジーの高いタンパク質が存在し，傷害や菌感染に誘導されるNaTI-IIやCBP20のプロセシングを行っていると思われる．これらのタンパク質はジャスモン酸やエチレンで誘導される．タバコにおけるVPEの発現調節にエチレンやジャスモン酸が関与しているか調べることにより，VPEがこれらのタンパク質をプロセシングしているかの一つの検討材料となると思われる．

プロモータ-GUSのデータより，γVPEの発現は維管束の付近にみられることが明らかになった．植物が傷害を受けたときに傷を受けた近傍の細胞にはカロースや二次代謝産物の蓄積がみられ，細胞壁を厚くする反応が起こる．また，傷害を受けた部位から水分の蒸発が起こるため，液胞の負担も大変なものと考えられる．γVPEの発現はこのような現象を反映しているかもしれない．

総合討論

液胞におけるVPEの役割

植物の液胞は種子ではタンパク質の蓄積，葉では細胞内に蓄積しているタンパク質の速やかな分解など種々の機能を果たしている．種子貯蔵型のVPEは貯蔵タンパク質の一つであるPV100のプロセシングに働き，機能的なプロテアーゼインヒビターと貯蔵タンパク質という異なる機能を持つタンパク質に変換すると考えられる．このことは，VPEが液胞タンパク質の機能発現に関わる特異的な役割を担っていることを示している．また，貯蔵組織型のヒマのVPEやタチナタマメのVPEがアスパラギン残基を特異的に認識し(Hara-Nishimura et al., 1993b, Abe et al., 1993)，ヒマのVPEが同様にアスパラギン酸を認識すること，PV100はアスパラギンおよびアスパラギン残基でプロセシングされていること，酵母に発現させたシロイヌナズナの栄養組織型のγVPEは種子貯蔵タンパク質のプロセシング部位を含むNG10をアスパラギン残基の後ろで切断されることから，栄養組織のアスパラギン部位でプロセシングされる液胞タンパク質がVPEにより成熟型へ変換される可能性が示唆される．しかし，ヒマの貯蔵組織型のVPEに比べて反応時間が3時間と長いことが気になる．今後，比活性と基質特異性を正確に測定する必要があると思われる．

ヒトのVPEホモログであるLegumainはアスパラギンのC末端側でペプチド結合を切断することが示されている．また，マウスのVPEホモログであるLegumainはリソソームにあることが示されている (Chen et al., 1998)．リソソームタンパク質であるCathepsin BやCathepsin Hがアスパラギンの後ろでプロセシングされるので，Legumainがこれらの酵素のプロセシングに働いていると考えられる．

最近，抗原提示B細胞においてLegumainがエンドサイトーシスにより取り込まれた抗原の部分的な消化に関与していることが示された (Manoury et al., 1998)．酵母はサイトゾルのタンパク質を液胞内にエンドサイトーシスにより取り込む自食作用を持っている (Baba et al., 1994)．植物でも飢餓状態のBY2細胞において自食作用があることが知られている (Moriyasu and Ohsumi, 1996)．栄養組織のγVPE，RD21Aは老化により誘導されるが，SAG2やSAG12も老化時に誘導されるプロテアーゼで，SAG2はアミノペプチダーゼであるAleurainのホモログである (Rogers et al., 1997)．ユリ (*Hemerocallis* x *hybrida*)の花弁の老化で発現してくるSEN102もシステインプロテアーゼである (Valpuesta et al., 1995)．また，トウモロコシ (*Zer mays*)の液胞のセリンエンドペプチダーゼは飢餓状態で誘導されてくる (James et al., 1996)．これらのことから老化時には液胞が活発に働き，エンドサイトーシスにより取り込まれた成分が分解酵素により速やかに分解される姿がうかがえる．

他にも，栄養組織のVPEは傷害で誘導され，シロイヌナズナの葉の排水組織に栄養組織型のVPEが特異的に発現してくること，根端に貯蔵組織型のVPEが発現してくること，オオムギの胚心に貯蔵組織型のVPEが胚発生の過程で発現してくること(Linnestad et al., 1998)より，それぞれの組織において液胞が機能的に働いていることを示している．今後，さらにVPEの基質となるタンパク質が，どの組織で，どのように存在しているか解析することにより液胞の機能が推定できると考えられる．

今後の展望

今後，シロイヌナズナのゲノム解析とともに液胞タンパク質の全貌が明らかになると考えられる．また，液胞タンパク質のプロテオーム解析が進めばプ

ロセシングのカスケードを明らかにすることが可能であると思われる．また，トウモロコシの種皮の形に変化を及ぼす*etched1*突然変異株は，栄養組織のVPEの変異体であることが示唆されている (Linnestad et al., 1998)．タンパク貯蔵型のVPEが発現するオオムギの胚心の細胞は胚発生の途中で分解していく．近年，植物でもクロマチンの濃縮，DNAの断片化など，動物のアポトーシスと同様の現象がアリューロン層，側根の形成部位，菌感染した細胞，葉の老化などで起こることが報告されてきている (Kosslak et al., 1997, Orzaez and Granell, 1997, Mittler et al.1997, Ryerson and Heath, 1996, Pennell and Lamb, 1997)．動物において，アポトーシスにはサイトゾルのプロテオリシスによるシグナル伝達が存在することが明らかにされているが，液胞に存在するVPEが細胞死のシグナル伝達に関わっているとは考えられず，細胞の成分の分解の過程で働いていると思われる．今後，VPEのアンチセンス，および突然変異体の表現型を解析することにより，植物の形態形成に関わるVPEの役割が明らかにされるであろう．

種子貯蔵タンパク質はほとんどが液胞に蓄積するため，VPEのターゲットとして適当である．しかし，栄養組織の液胞タンパク質はその量が大変少ないため，VPEのターゲットをタンパク質から探ることは非常に難しいと思われる．近年，システインプロテアーゼであるCaspase 3の基質を酵母のtwo hybrid スクリーニングする方法が確立された (Kamada et al., 1998)．この方法では，結合した基質が分解されないようにプロテアーゼの活性部位に変異を入れてある．このような方法が植物での解析においても有効であると思われる．

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謝　辞

本研究を遂行するにあたり，本当に忙しい中終始適切なご指導を賜り，便宜を計っていただきました，西村幹夫先生，西村いくこ先生に心から感謝いたします．また，研究において適切な助言を頂いた嶋田知生先生，林誠先生をはじめとする細胞機構研究部門の職員，学生のみなさまに深く感謝いたします．

また，カボチャやシロイヌナズナの面倒を見て下さった難波さん，質量スペクトログラフィーやアミノ酸シーケンスを行っていただいた牧野さんに深く感謝いたします．

報 文 目 録

Yamada, K., Shimada, T., Kondo, M., Nishimura, M., Hara-Nishimura, I. (1999)

Multiple functional proteins are produced by cleaving Asn-Gln bonds of a single precursor by vacuolar processing enzyme.

J. Biol. Chem. **274**, 2563-2570

Kinoshita, T*., Yamada, K*., Hiraiwa, N., Kondo, M., Nishimura, M., Hara-Nishimura, I. (1999) *The first two authors contributed equally in this study.

Vacuolar processing enzyme is induced in the lytic vacuoles of vegetative tissues during senescence and under stressed conditions.

Plant J. **19**, 1-11

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 4, Issue of January 22, pp. 2563–2570, 1999
Printed in U.S.A.

Multiple Functional Proteins Are Produced by Cleaving Asn-Gln Bonds of a Single Precursor by Vacuolar Processing Enzyme*

(Received for publication, May 15, 1998, and in revised form, September 10, 1998)

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Precursor-accumulating vesicles mediate transport of the precursors of seed proteins to protein storage vacuoles in maturing pumpkin seeds. We isolated the precursor-accumulating vesicles and characterized a 100-kDa component (PV100) of the vesicles. Isolated cDNA for PV100 encoded a 97,310-Da protein that was composed of a hydrophobic signal peptide and the following three domains: an 11-kDa Cys-rich domain with four CXXXC motifs, a 34-kDa Arg/Glu-rich domain composed of six homologous repeats, and a 50-kDa vicilin-like domain. Both immunocytochemistry and immunoblots with anti-PV100 antibodies showed that <10-kDa proteins and the 50-kDa vicilin-like protein were accumulated in the vacuoles. To identify the mature proteins derived from PV100, soluble proteins of the vacuoles were separated, and their molecular structures were determined. Mass spectrometry and peptide sequencing showed that two Cys-rich peptides, three Arg/Glu-rich peptides, and the vicilin-like protein were produced by cleaving Asn-Gln bonds of PV100 and that all of these proteins had a pyroglutamate at their NH_2 termini. To clarify the cleavage mechanism, *in vitro* processing of PV100 was performed with purified vacuolar processing enzyme (VPE). Taken together, these results suggested that VPE was responsible for cleaving Asn-Gln bonds of a single precursor, PV100, to produce multiple seed proteins. It is likely that the Asn-Gln stretches not only provide cleavage sites for VPE but also produce aminopeptidase-resistant proteins. We also found that the Cys-rich peptide functions as a trypsin inhibitor. Our findings suggested that PV100 is converted into different functional proteins, such as a proteinase inhibitor and a storage protein, in the vacuoles of seed cells.

In higher plants, proprotein precursors of most seed proteins are synthesized on the rough endoplasmic reticulum and are then transported to protein storage vacuoles in maturing seed cells (1–3). We have shown that the vesicles with a density of 1.24 g/cm^3 mediate the delivery of proprotein precursors of seed proteins to the vacuoles (4–6). We have succeeded in isolation of the vesicles from maturing pumpkin seeds and have shown that they contained a large amount of precursors of various seed proteins, including 11S globulin and 2S albumin (7). Thus, these vesicles were designated precursor-accumulating (PAC)[1] vesicles. Recently, we have found that the PAC vesicles contain a type I integral membrane protein with epidermal growth factor-like motifs and have shown that the membrane protein binds to peptides derived from the 2S albumin precursor (8). The membrane protein of the PAC vesicles might function as a sorting receptor for seed protein precursors to the vacuoles.

Just after arriving at the vacuoles, the precursor proteins are converted into their respective mature forms by proteolytic cleavages (6, 9). The posttranslational cleavages occur at the carbonyl sides of Asn residues in precursors of various seed proteins of different plants, including storage proteins, lectins, and toxins, as reviewed by Hara-Nishimura *et al.* (10). We have found an enzyme responsible for maturation of these seed proteins and have designated it vacuolar processing enzyme (VPE) (11, 12). VPE recognizes exposed Asn residues on the molecular surface of the precursor proteins and then cleaves the peptide bonds at the carbonyl sides of the Asn residues (6). The VPE-mediated processing system plays a crucial role in maturation of various seed proteins in protein storage vacuoles.

Our previous study showed that VPE homologs can be separated into two subfamilies: one specific to seeds and the other specific to vegetative organs (13, 14). This is consistent with the fact that the plant vacuoles are classified into two types, protein storage vacuoles in seeds and lytic vacuoles in vegetative organs. A VPE-mediated processing system similar to that in protein storage vacuoles is involved in maturation of vacuolar proteins in lytic vacuoles (15). Vegetative VPE might be responsible for conversion of inactive precursor into their mature active proteins, such as proteinase inhibitors and hydrolytic enzymes (15). Similarly, it is likely that seed VPE also plays a role in the activation of some functional proteins in seeds.

Each of most precursor proteins is composed of a functional domain and an NH_2- and/or COOH-terminal propeptide(s), except for a precursor protein of proteinase inhibitors of tobacco that is processed into five homologous inhibitors and an NH_2-terminal propeptide (16). On the other hand, it is not known whether multiple vacuolar proteins with distinct functions are derived from a single precursor. In this study, we demonstrated that a 100-kDa component of the PAC vesicles (PV100) is converted into multiple proteins with a pyroglutamate at their NH_2 termini by cleaving Asn-Gln bonds of PV100 by the action of VPE, after arrival at protein storage vacuoles. We show here a unique mechanism for vacuolar processing at Asn-Gln cas-

* This work was supported by Grants-in-Aid 10440244, 10163242, and 09267241 for the Research for the Future Program (JSPS-RFTF96L00407) from the Japan Society for the Promotion of Science and for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB019195.

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[1] The abbreviations used are: PAC, precursor-accumulating; PV100, a 100-kDa component of PAC vesicles; VPE, vacuolar processing enzyme; BAPA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide HCl; bp, base pair; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

settes in the precursor sequence to produce aminopeptidase-resistant proteins in the plant vacuoles.

EXPERIMENTAL PROCEDURES

Plant Materials—Pumpkin (*Cucurbita maxima* cv. Kurokawa Amakuri Nankin) seeds were purchased from Aisan Shubyo Seed Co. (Nagoya, Japan). For isolation of PAC vesicles and immunocytochemical analysis, pumpkin seeds were planted in the farm of Nagoya University during the summer season, and cotyledons of the maturing seeds, freshly harvested 22–28 days after pollination, were collected.

Isolation of PAC Vesicles—PAC vesicles were isolated from pumpkin cotyledons at the middle stage of seed maturation essentially as described previously (7). The cotyledons (15 g) were homogenized in a solution (7 ml/g fresh weight of cotyledons) of 20 mM sodium pyrophosphate (pH 7.5), 1 mM EDTA, and 0.3 M mannitol with an ice-chilled mortar and pestle, and the homogenate was filtered through cheesecloth. The filtrate was centrifuged at 3000 × *g* for 15 min and the supernatant was centrifuged again at 8000 × *g* for 20 min at 4 °C. The pellet was suspended in 1 ml of 10 mM Hepes-KOH (pH 7.2), 1 mM EDTA, and 0.3 M mannitol. The suspension was layered on a solution of 28% Percoll (Amersham Pharmacia Biotech) in 10 mM Hepes-KOH (pH 7.2), 1 mM EDTA, and 0.3 M mannitol on a cushion of 2 ml of 90% Percoll. Centrifugation was at 40,000 × *g* for 30 min at 4 °C. The vesicle fraction was centrifuged again in a self-generated Percoll gradient. The resulting vesicle fraction was washed in the above-described Hepes-KOH buffer and used for immunoelectron microscopy and immunoblot analysis.

Isolation of Protein Storage Vacuoles and Purification of Proteins Derived from PV100—Protein storage vacuoles (protein bodies) were isolated from dry pumpkin seeds (50 g) by a nonaqueous isolation method, as described previously (17). Isolated protein storage vacuoles were burst in 100 ml of 10 mM Tris-MES (pH 6.5), 0.1 M sucrose, 1 mM EDTA; sonicated; and then centrifuged at 100,000 × *g* for 1 h at 4 °C to remove insoluble proteins and membranes as a pellet, as described previously (18). Ammonium sulfate was added to the supernatant solution to a concentration of 30% saturation. The mixture was incubated for 1 h at 5 °C and then centrifuged at 200,000 × *g* for 15 min. The ammonium sulfate concentration of the supernatant was then increased to 100% saturation, and the incubation and centrifugation steps were repeated. The precipitate was suspended in 4 ml of a solution of 25 mM sodium acetate (pH 5.5) and 5 mM EDTA and then applied to an Econo-*Pac*10 DG column (Bio-Rad) to remove ammonium sulfate. The preparation was used as the matrix fraction of the protein storage vacuoles.

The matrix fraction was found to contain a large amount of the 4–6-kDa proteins and the 50-kDa protein that were derived from PV100 by posttranslational cleavage. To purify the 4–6-kDa proteins, the matrix fraction was applied to either a reverse phase column (μRPC C2/C18 PC 3.2/3) on a SMART system (Amersham Pharmacia Biotech) or a reverse phase column (μRPC C2/C18 ST 4.6/100) on an ÄKTA system (Amersham Pharmacia Biotech). Elution was carried out with a gradient starting from 0.065% trifluoroacetic acid in distilled water to 0.05% trifluoroacetic acid in acetonitrile, at a flow rate of 200 μl/min for SMART and at a rate of 500 μl/min for ÄKTA. Each fraction was subjected to immunoblot analysis, mass spectrometry, and digestion by pyroglutamate aminopeptidase followed by automatic Edman degradation, as described below, to determine the molecular structures of these proteins. We also measured trypsin inhibitory activity in the fractions to demonstrate the function of the PV100-derived small proteins.

Determination of NH_2-terminal and Internal Amino Acid Sequences—The PAC vesicles and the matrix fraction of the protein storage vacuoles were subjected to SDS-PAGE, and then the separated proteins were transferred electrophoretically to an Immobilon-P membrane (0.22 mm) (Nihon Millipore Ltd., Tokyo, Japan). After staining of proteins on the blot with Coomassie Blue, the band corresponding to either PV100 or the 50-kDa protein was cut out from the blot and subjected to automatic Edman degradation on a peptide sequencer (model 492, Applied Biosystems Inc.).

To determine the internal sequence of PV100, the separated proteins of the PAC vesicles were stained with Coomassie Blue, and the band corresponding to PV100 on the SDS gels was cut out from the gel, as described previously (19). The gel piece was incubated with 5 μg of V8 protease (Sigma) by the method of Cleveland *et al.* (20). After Tricine-SDS-PAGE (21), the separated peptides were transferred to an Immobilon-P membrane and subjected to automatic Edman degradation.

Digestion with Pyroglutamate Aminopeptidase Followed by Edman Degradation—Each 3 μg of the purified 4–6-kDa proteins of the protein storage vacuoles was digested with 0.3 μg of pyroglutamate aminopeptidase (Boehringer Mannheim) in 20 μl of solution of 0.1 M sodium phosphate (pH 8.0), 5% glycerol, 5 mM dithiothreitol, and 1 mM EDTA for 6 h at 50 °C. The digests were directly subjected to automatic Edman degradation.

Purification of VPE—Protein storage vacuoles (protein bodies) were isolated from castor bean endosperm by a nonaqueous isolation method, as described above. VPE was purified from the soluble fraction of the protein storage vacuoles by using ammonium sulfate precipitation and Con-A Sepharose and MonoS column chromatographies, as described previously (11).

In Vitro Processing by VPE—The PAC vesicles (60 μg of proteins) were incubated with the purified VPE in a solution of 50 mM sodium acetate buffer (pH 5.5) and 50 mM dithiothreitol for 15 h at 37 °C. The reaction was subjected to SDS-PAGE and the separated proteins on the gels were transferred electrophoretically to a polyvinylidene difluoride membrane (0.22 μm) (Nihon Millipore Ltd.). The band corresponding to the 50-kDa protein was cut out from the blot to determine the NH_2-terminal sequence. The membrane piece was incubated with 0.5% polyvinylpyrrolidone for 30 min at 37 °C, followed by digestion by 2.5 μg of pyroglutamate aminopeptidase (Boehringer Mannheim) in 40 μl of solution of 0.1 M sodium phosphate (pH 8.0), 5% glycerol, 5 mM dithiothreitol, and 10 mM EDTA at room temperature for 18 h. After the removal of a pyroglutamate at the NH_2 terminus, the 50-kDa protein on the membrane piece was subjected to automatic Edman degradation on a peptide sequencer (model 492, Applied Biosystems Inc.).

Mass Spectrometry—To determine the exact molecular mass of the 4–6-kDa proteins of the protein storage vacuoles, each fraction that was separated on a SMART system as described above was applied to an API 300 triple quadrupole mass spectrometer (PE SCIEX, Foster City, CA) in positive ion detection mode, equipped with ion spray interface. Samples were dissolved in 0.1% formic acid and 50% acetonitrile and then delivered at 3 μl/min. The sprayer was held at a potential of 4.5 kV. Orifice potential was maintained at 25 V.

Assay of Trypsin Inhibitory Activity—Trypsin inhibitory activity was assayed essentially as described by Cechova (22). α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPA) was used as a substrate of trypsin. One of the PV100-derived small proteins, C2 peptide, purified by a reverse phase chromatography, was dissolved in a solution of 0.1 M Tris-HCl (pH 8.0) and 25 mM $CaCl_2$. After preincubation of the C2 peptide (0–2.4 nmol) with 10 μg of trypsin (Sigma) in a 676 μl of 0.1 M Tris-HCl (pH 8.0) and 25 mM $CaCl_2$ at room temperature for 30 min, 333 μl of a substrate BAPA solution (1 mg/ml) was added to the mixture to start the reaction. After incubation of the mixture at room temperature for 30 min, 100 μl of acetic acid was added to stop the reaction. The residual enzyme activity was measured at 405 nm. The amount of the C2 peptide was estimated from the absorbance at 280 nm, and the molar absorption coefficient at 280 nm of the C2 peptide was computed.

Isolation of cDNA for PV100 and Determination of Nucleotide Sequence—A cDNA library in pBluescript II SK+ (Stratagene, La Jolla, CA) was constructed with the poly(A)$^+$ RNA from maturing pumpkin cotyledons, as described previously (23). Four degenerate primers, 1F (5′-GG(A/C/G/T)GC(A/C/G/T)GG(A/C/G/T)GT(A/C/G/T)GA(C/T)CA-3′), 2F (5′-CA(C/T)GA(C/T)GG(A/C/G/T)TG(C/T)GT(A/C/G/T)-3′), 3R (5′-GG(A/G/T)AT(A/C/G/T)GTCAT(A/C/G/T)AC(A/G)TC-3′), and 4R (5′-TA(G/A)TC(T/C)TT(G/A)AA(T/C)TC(A/C/G/T)CC)-3′), were designed on the basis of the NH_2-terminal and internal amino acid sequences of PV100 and synthesized on a DNA synthesizer (model 394, Applied Biosystems Inc., Foster City, CA). Polymerase chain reaction was performed using a set of the 1F and 4R primers and the cDNA library as a template. A 1454-bp DNA was amplified. A second polymerase chain reaction using a set of 2F and 3R primers and the 1454-bp DNA was performed to amplify a 1340-bp DNA. The 1340-bp DNA was inserted into the T-vector to confirm the nucleotide sequence. The 1340-bp DNA was labeled with [γ-^{32}P]dCTP and Megaprime DNA labeling systems (Amersham Pharmacia Biotech). The cDNA library was screened by colony hybridization using the ^{32}P-labeled DNA as a probe. The isolated cDNA lacked an initiation codon. Subsequently, we amplified DNAs covering the 5′ region of PV100 cDNA using a 5′-Full RACE Core Set (Takara, Tokyo, Japan). Two identical clones were amplified, and the nucleotide sequences were overlapped with the isolated cDNA sequence that lacked an initiation codon.

DNA sequencing was performed with a DNA sequencer (model 377, Applied Biosystems Inc.) and −21M13 forward and M13 reverse fluorescent primers in accordance with the manufacturer's directions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (Gene Works, IntelliGenetics, Mountain View, CA). The hydrophobicity profile of the amino acid sequences was com-

puted by application of the algorithm of Kyte and Doolittle (24), with a window size of 10 residues. A homology plot was computed with the PAM-250 algorithm (25).

Preparation of Specific Antisera—The isolated PAC vesicles were subjected to SDS-PAGE on a 12.5% polyacrylamide gel with subsequent staining with Coomassie Blue. The band corresponding to the PV100 protein with a molecular mass of 100 kDa was cut out from the gel and gently shaken in phosphate-buffered saline for several hours. The gel was emulsified with complete Freund's adjuvant and injected subcutaneously into a rabbit. After 3 weeks, two booster injections with incomplete adjuvant were given at 7-day intervals. One week after the booster injections, blood was drawn, and the antiserum was prepared.

Immunoblot Analysis—Both the PAC vesicles and protein storage vacuoles were subjected to SDS-PAGE followed by either Coomassie Blue staining or immunoblotting. The purified 4–6-kDa proteins of the protein storage vacuoles were also subjected to immunoblot analysis. The immunoblot was performed essentially as described previously (18). The separated proteins on gels were transferred electrophoretically to a polyvinylidene difluoride membrane (0.22 μm) (Nihon Millipore Ltd., Tokyo, Japan). The membrane blot was incubated overnight with anti-PV100 antibodies that were diluted 2000-fold in a solution of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% (v/v) Tween 20, and 3% (w/v) skim milk. Alkaline phosphatase-conjugated antibodies (Cappel, West Chester, PA) and horseradish peroxidase-conjugated antibodies (Amersham Pharmacia Biotech) that were raised in goat against rabbit IgG were diluted 2000-fold and used as second antibodies.

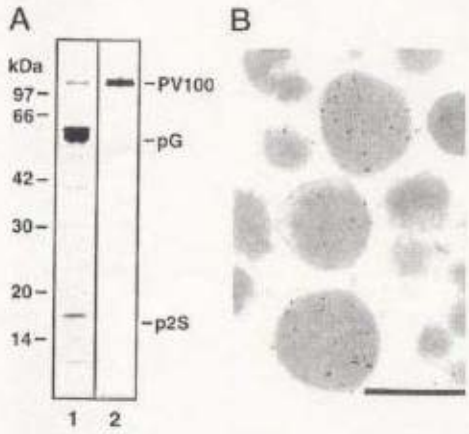


FIG. 1. **PV100 is one of the major proteins in the PAC vesicles from maturing pumpkin seeds.** *A*, isolated PAC vesicles were subjected to SDS-PAGE and subsequent staining with Coomassie Blue (*lane 1*) or immunoblot with anti-PV100 antibodies (*lane 2*). *pG* and *p2S* represent proprotein precursors of 11S globulin and 2S albumin, respectively. The molecular mass of each marker protein is given on the *left* in kDa. PAC vesicles were isolated from the cotyledons at the middle stage of seed maturation of pumpkin. *B*, immunogold labeling of the isolated PAC vesicles with anti-PV100 antibodies. *Bar*, 500 nm.

Immunoelectron Microscopy—Maturing pumpkin seeds were freshly harvested. The cotyledons were vacuum-infiltrated for 1 h with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.06 M sucrose in 0.05 M cacodylate buffer (pH 7.4). The tissues were then cut into slices of less than 1 mm in thickness with a razor blade and treated for another 2 h with freshly prepared fixative. The isolated PAC vesicles were fixed in 4% paraformaldehyde, 1% glutaraldehyde, 0.3 M mannitol, 1 mM EDTA, and 10 mM Hepes-KOH (pH 7.2) for 1 h at 4 °C. The samples were dehydrated in a graded dimethylformamide series at −20 °C and embedded in LR white resin (London Resin Co. Ltd., Basingstoke, Hampshire, UK). Immunogold labeling procedures were essentially the same as those described previously (26), except for the use of the anti-PV100 antibodies that were diluted 1000-fold in blocking solution (1% bovine serum albumin in phosphate-buffered saline). Protein A-gold (15 nm) (Amersham Pharmacia Biotech) was diluted 40-fold and used. The ultrathin sections were examined with a transmission microscope (model 1200EX) (JEOL, Tokyo, Japan) at 80 kV.

RESULTS

PV100 Is a 100-kDa Protein Component of PAC Vesicles—We have shown that the PAC vesicles are responsible for the intracellular transport of precursors of major seed proteins, including 11S globulin and 2S albumin, to protein storage vacuoles in maturing pumpkin seeds (4, 6–8). The PAC vesicles were highly purified from cotyledons of maturing pumpkin seeds. Electron microscopy revealed that each PAC vesicle contained an electron-dense core with a diameter of 300–500 nm and that the isolated vesicles were barely contaminated by other cellular components (Fig. 1*B*). Fig. 1*A* (*lane 1*) shows the protein components of the vesicles that were separated on an SDS-gel with Coomassie Blue staining. Three major proteins were found in the PAC vesicle fraction. Two of them have been shown to correspond to proglobulin, a proprotein precursor of 11S globulin (4), and to pro2S albumin, a proprotein precursor of 2S albumin (6), as indicated by *pG* and *p2S* in Fig. 1*A* (*lane 1*), respectively. The third component of the PAC vesicles, with

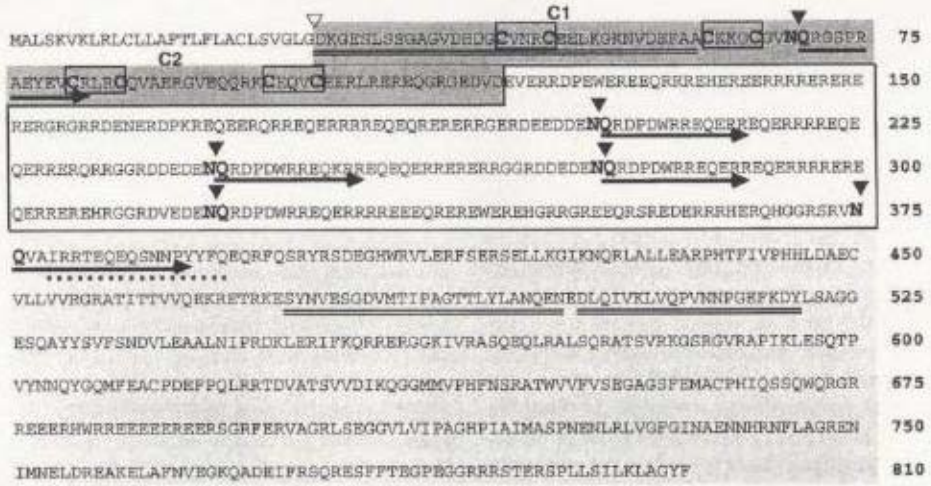


FIG. 2. **Deduced amino acid sequence from a cDNA that encodes PV100.** Isolated cDNA encodes a 97,310-Da protein of 810 amino acids, which consists of a hydrophobic signal peptide followed by the PV100 sequence. The NH_2-terminal sequence and two internal sequences of PV100 that were determined are indicated by *double underlining*. An *open triangle* indicates a cleavage site of a signal peptide. The PV100 sequence was divided into three domains: an 11-kDa Cys-rich domain (indicated by *dark shading*) with four CXXXC motifs (enclosed in the *small boxes*), a 34-kDa Arg/Glu-rich domain (enclosed in the *large box*), and a 50-kDa vicilin-like domain (indicated by *light shading*). The *arrows* indicate the determined NH_2-terminal sequences of PV100-derived mature proteins that had been digested by pyroglutamate aminopeptidase (see Figs. 6 and 8), and a *dotted line* indicates the NH_2-terminal sequence of the vicilin-like protein from dry seeds. *Boldfaced NQ* (Asn-Gln) stretches, marked with a *closed triangle*, represent posttranslational processing sites to produce multiple seed proteins, each with a pyroglutamate at its NH_2 terminus. The nucleotide sequence has been submitted to the DNA Data Bank of Japan and GenBank™ with the accession number AB019195.

a molecular mass of 100 kDa, was designated PV100.

For immunochemical characterization of PV100, polyclonal antibodies were raised against the PV100 protein. An immunoblot of the PAC vesicles with the anti-PV100 antibodies showed that the antibodies specifically recognized PV100 on the blot, as shown in Fig. 1*A* (*lane 2*). Immunoelectron microscopy of the isolated PAC vesicles with the anti-PV100 antibodies shows that gold particles are inside the PAC vesicles (Fig. 1*B*). These results indicate that PV100 is localized in the PAC vesicles together with proproteins of the major seed proteins, 11S globulin and 2S albumin.

PV100 Is Composed of Three Domains—The next issue to be solved was the molecular structure of PV100 to clarify the manner of posttranslational cleavage. We determined the amino acid sequences of the NH_2 terminus and two internal fragments of PV100 and then isolated a cDNA with a 4.3-kb insert from the library of maturing pumpkin seeds based on the amino acid sequences. The cDNA encoded a 97,310-Da protein of 810 amino acids, as shown in Fig. 2. The determined NH_2-terminal amino acid sequence, DKGESLSSGAGVDHDGCVNRCEELKGXNVDEFAA (*X*, not determined), and the two internal sequences, *X*YNVESGDVMTIPAGTTLYLANQEN and DLQIVKLVQPVNNPGEFKDY, were found in the deduced sequence (Fig. 2, *double-underlined* sequences), indicating that this clone was a cDNA for PV100. The deduced primary structure of the PV100 protein was composed of a hydrophobic signal peptide followed by the PV100 sequence. The NH_2-terminal sequence of PV100 revealed that the signal peptide is cleaved off co-translationally on the carbonyl side of Gly^{27}, as indicated in Fig. 2 (*open triangle*). The cleavage site was consistent with that predicted by application of the rules of Von Heijne (27).

The PV100 sequence was composed of three domains: an 11-kDa Cys-rich domain, a 34-kDa Arg/Glu-rich domain, and a 50-kDa vicilin-like domain, as shown in Fig. 2. The Cys-rich domain contained four CXXXC motifs (Fig. 2, *boxes*) of two Cys residues separated by three other amino acids. The 50-kDa domain (Fig. 2, *light shading*) exhibited a 30–35% identity in amino acids to the vicilin homologs, pea vicilin (28), soybean β-conglycinin (29), and jack bean canavalin (30). Precursors of vicilin homologs of pea, soybean, and jack bean are composed of a signal peptide followed by a vicilin domain, whereas the precursors of cacao vicilin (31) and upland cotton α-globulin-A (32) have six and four CXXXC motifs, respectively, preceding a vicilin domain. However, the amino acid sequences around the CXXXC motifs of pumpkin, cacao, and cotton exhibited a very low similarity to each other.

The Arg/Glu-rich domain of PV100 is composed of 37 mol % Arg and 27 mol % Glu, as shown in Fig. 2 (*large box*). It should be noted that this domain is unique to pumpkin PV100. A homology plot of PV100 shows that six homologous repeats are found in the Arg/Glu-rich domain, as shown in Fig. 3*A*. An alignment revealed that the six homologous repeats were separated by Asn-Gln (Glu) sequences (Fig. 3*B*). The six repeats rich in Arg and Glu were designated RE1–RE6 in order from the NH_2 terminus. They show a sequence homology to pumpkin basic peptide of 36 amino acids that was isolated from pumpkin (*C. maxima* cv. Mexican-papitas) seeds (33). In particular, the 36-amino acid form of RE3 that was found in the seeds could be identical to the pumpkin basic peptide (Fig. 3*C*, discussed below).

Multiple Seed Proteins That Are Derived from PV100 Are Accumulated in Protein Storage Vacuoles—Our previous works have shown that both proglobulin and pro2S albumin are transported from the PAC vesicles to the protein storage vacuoles and then are converted into their respective mature forms (4, 6). This raises the question of whether PV100 is also incorporated into the protein storage vacuoles. Immunocytochemistry of the maturing pumpkin seeds with the anti-PV100 antibodies answered this question. Electron-dense PAC vesicles with diameters of 300–500 nm and protein storage vacuoles composed of crystalloids of 11S globulin and the matrix were observed in the cells, as shown in Fig. 4*A*. Gold particles can be seen distributed in the vacuolar matrix region, the PAC vesicles, and the rough endoplasmic reticulum. In contrast, none of the vacuolar crystalloid, the lipid bodies, the mitochondria, or the cell wall was labeled with gold particles. These results suggested that PV100, which is synthesized on rough endoplasmic reticulum, is transported to PAC vesicles and then to protein storage vacuoles.

The molecular structure of PV100 implies that multiple seed proteins are derived from PV100. The next question is whether PV100 is processed to make such seed proteins in the protein

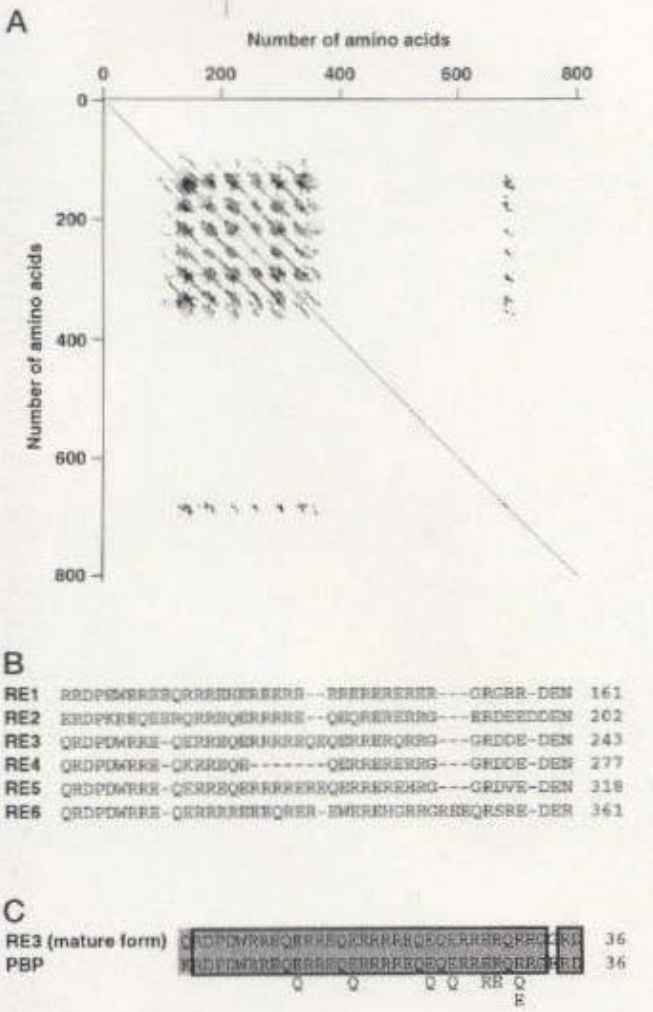


FIG. 3. **Homology plot of PV100 and an amino acid alignment of the six homologous repeats in the Arg/Glu-rich domain.** *A*, a homology plot was performed with the PAM-250 algorithm (25) with a window of 10 residues. Each pair of windows that exhibited more than 35% identity in amino acids is indicated by a *dot* in the matrix. Six homologous repeats were found in the Arg/Glu-rich domain. *B*, Asn-Gln/Glu bonds separate the Arg/Glu-rich domain into six repeats (see Fig. 9). The six Arg/Glu-rich repeats that were designated RE1 to RE6 in order from the NH_2 terminus were aligned. Numbers on the *right* side of each sequence refer to the positions of the amino acids starting from the initiation Met. *C*, the mature RE3 peptide (see Fig. 6*B*) was aligned with the sequence of pumpkin basic peptide, where the second and the third possible amino acids are also shown, as reported by Naisbitt *et al.* (33). Both peptides are composed of 36 amino acids, as indicated on the *right* side of each sequence. *Boxes* enclose identical amino acids, and *shading* indicates homologous amino acids.

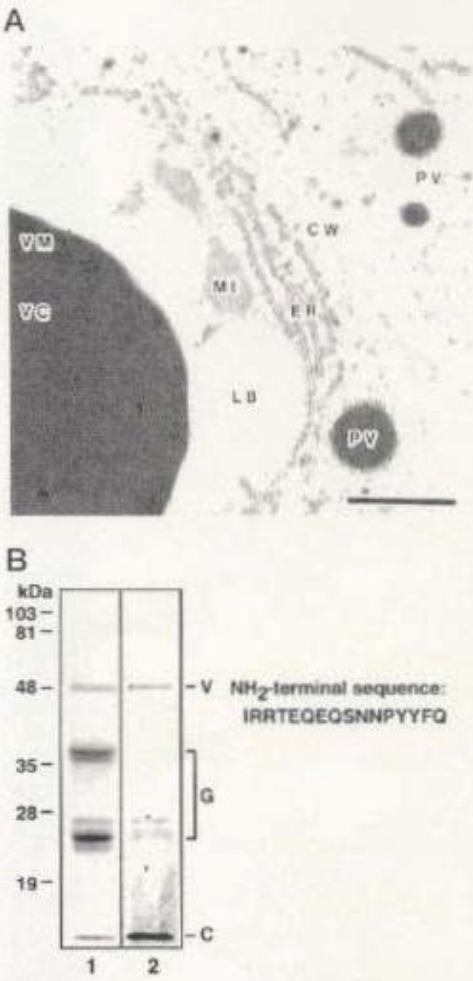


FIG. 4. **PV100-derived proteins are localized in protein storage vacuoles in pumpkin seeds.** *A*, immunoelectron micrograph of maturating pumpkin seeds after staining with anti-PV100 antibodies. Gold particles were distributed in the PAC vesicles (*PV*), the matrix region (*VM*) of protein storage vacuoles and ER. *VC*, vacuolar crystalloid composed of 11S globulin; *Mt*, mitochondrion; *LB*, lipid body; *CW*, cell wall. *Bar*, 1 μm. *B*, isolated protein storage vacuoles (protein bodies) from dry pumpkin seeds were subjected to SDS-PAGE and subsequent staining with Coomassie Blue (*lane 1*) or immunoblot with anti-PV100 antibodies (*lane 2*). PV100-derived proteins, the 50-kDa vicilin-like protein (*V*) and a ~6-kDa C2 peptide (*C*) were detected on the blot. The determined NH_2-terminal sequence of the 50-kDa vicilin-like protein is shown. *G* represents the 11S globulin. The molecular mass of each marker protein is given on the *left* in kDa.

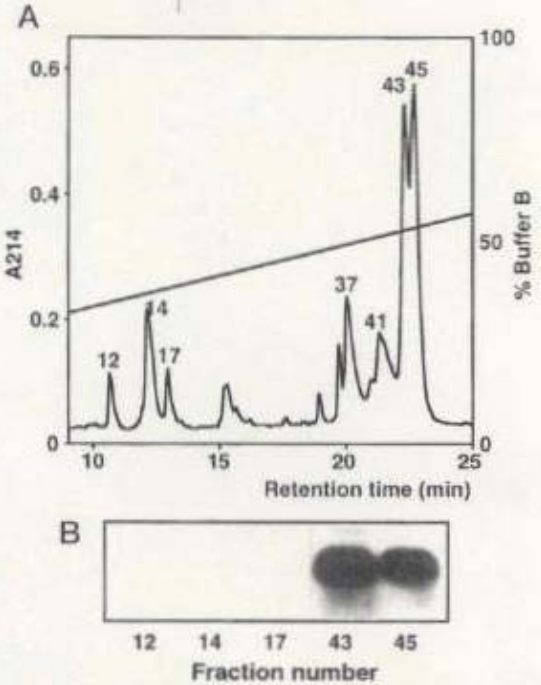


FIG. 5. **An HPLC profile of PV100-derived peptides from the protein storage vacuoles.** *A*, soluble fraction of the protein storage vacuoles that contained the PV100-derived peptides was applied to a reverse phase column. Elution was carried out with a gradient starting from 0.065% trifluoroacetic acid in distilled water to 0.05% trifluoroacetic acid in acetonitrile. Chromatography was monitored in terms of absorbance at 214 nm. *B*, immunoblot analysis of each peak fraction with anti-PV100 antibodies.

storage vacuole. To answer this question, the protein storage vacuoles were isolated from dry pumpkin seeds and subjected to SDS-PAGE followed by immunoblot analysis, as shown in Fig. 4*B*. The vacuoles contained a large amount of 11S globulin (Fig. 4*B*, *lane 1*). On the immunoblot with anti-PV100 antibodies, two bands, corresponding to a 50-kDa protein and a <10-kDa small protein(s), were detected (Fig. 4*B*, *lane 2*). The NH_2-terminal sequence of the 50-kDa protein was IRRTEQEQSNNPYYFQ, which corresponds to a sequence starting from the fourth amino acid of the vicilin-like domain, as indicated in Fig. 2, *dotted line* (discussed below). These results suggested that the 50-kDa vicilin-like protein and <10-kDa small protein(s) were produced from PV100 and were accumulated in the protein storage vacuoles.

To identify the <10-kDa small protein(s), soluble proteins of the protein storage vacuoles were separated by HPLC, as shown in Fig. 5*A*. Each peak fraction of the HPLC was subjected to both mass spectrometry and automatic Edman degradation (Fig. 6). Fractions 37 and 41 contained the known *C. maxima* trypsin inhibitor (34), which is not related to PV100. The NH_2 termini of all proteins of fractions 12, 14, 17, 43, and 45 were blocked. When digested by pyroglutamate aminopeptidase, each protein gave an NH_2-terminal amino acid sequence that was consistent with the sequence starting from the second residues of the respective small protein derived from PV100, as indicated in Figs. 2 and 6*B* (*arrow*). Fractions 12, 14, and 17 corresponded to RE4, RE3, and RE5 of the Arg/Glu-rich domain, respectively, and fractions 43 and 45 corresponded to the latter half (C2) of the Cys-rich domain (Fig. 6). Interestingly, all of these small proteins had a pyroglutamate at their NH_2 termini. It should be noted that an Asn residue always preceded all Gln residues to be converted into a pyroglutamate, as indicated in Fig. 2 (*boldfaced NQ* in the PV100 sequence) (discussed below).

The observed molecular masses of fractions 12, 14, and 17 showed good agreement with the theoretical masses of sequence d of RE4, sequence c of RE3, and sequence e of RE5, respectively, each of which has a pyroglutamate at the NH_2 terminus and an Asp residue at the COOH terminus (Fig. 6). Thus, two steps of processing might be required to produce the mature forms of RE peptides from PV100: the first is cleavage at Asn-Gln bonds of PV100, and the second is trimming 2 or 5 amino acids off at the COOH termini of RE intermediates. All of the mature peptides of the Arg/Glu-rich RE3, RE4, and RE5 found in seeds are basic ones with estimated pIs of 11.90, 11.54, and 10.20, respectively. This is in contrast to the neutral pIs of RE intermediates before trimming their COOH-terminal few amino acids.

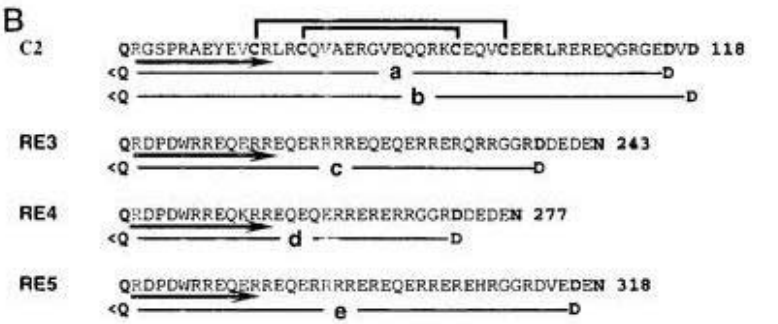
The observed molecular masses of fractions 43 and 45 also showed good agreement with the theoretical masses of sequences a and b of C2 from the Cys-rich domain, respectively, indicating that each sequence has a pyroglutamate at the NH_2 terminus and two intramolecular disulfide bonds (Fig. 6). The

FIG. 6. **Molecular structures of PV100-derived peptides from the protein storage vacuoles.** *A*, molecular masses of the peptides in each HPLC fraction, as shown in Fig. 5, were determined by mass spectrometry. Theoretical molecular masses of RE3, RE4, and RE5 that had a pyroglutamate (<Q) at their NH_2 termini are consistent with the observed values. The number of disulfide bonds in the C2 peptide was determined to be 2. *B*, primary structures of peptide components of fractions 12, 14, 17, 43, and 45 were determined to be *sequence d* (RE4), *sequence c* (RE3), *sequence e* (RE5), and *sequences a* and *b* (C2), respectively. The determined NH_2-terminal sequences after digestion by pyroglutamate aminopeptidase are indicated by *arrows* below the respective sequences. Numbers on the *right* side of each sequence refer to the positions of the amino acids starting from the initiation Met of PV100. The disulfide bridges were deduced from the data of buckwheat trypsin inhibitor that exhibits a characteristic similar to the C2 peptide and has two CXXXC motifs and two disulfide bridges (35).

A

Fraction number	Observed mass	Name of peptide	Sequence in (B)	NH_2 terminus	Number of disulfide bonds	Theoretical mass
12	3876.52±0.71	RE4	d	<Q	0	3877.00
14	4888.00±0.50	RE3	c	<Q	0	4888.03
17	5242.17±0.90	RE5	e	<Q	0	5241.34
43	5615.54±0.70	C2	a	<Q	2	5615.02
45	5829.90±0.80	C2	b	<Q	2	5829.23

B



disulfide bridges are deduced from the data of buckwheat trypsin inhibitor, an allergenic protein, that exhibits a similar characteristic to the C2 peptide and has two CXXXC motifs and two disulfide bridges (35).

To explore function of the C2 peptide, we examined trypsin inhibitory activity of the C2 peptide using BAPA as a substrate of trypsin. The C2 peptide was highly purified. Mass spectrometry showed that the final preparation of the C2 peptide used for the assay was not contaminated by *C. maxima* trypsin inhibitor. We found that the C2 peptide had an inhibitory activity against trypsin, as shown in Fig. 7. Ten μg of trypsin was completely inhibited by 1.2 nmol of C2 peptide. The C2 peptide of 49 amino acids exhibits an 18% identity in amino acids to buckwheat trypsin inhibitor of 51 amino acids. In contrast to the low identity between the two sequences, they have a similar characteristic in the presence of two CXXXC motifs in their sequences. The reactive site of buckwheat trypsin inhibitor for trypsin was reported to be Arg^{19}, between the two CXXXC motifs (35). The C2 peptide conserves Arg^{21}, between the two CXXXC motifs (Fig. 6*B*), and the residue might be the reactive site for trypsin (discussed below).

None of RE1, RE2, RE6, or the former half (C1) of the Cys-rich domain was detected in protein storage vacuoles. They might be degraded in the vacuoles during seed maturation (discussed below). These results indicated that ~6-kDa C2, ~5-kDa RE3, ~4-kDa RE4, and ~5-kDa RE5 are accumulated in the protein storage vacuoles. Fig. 5*B* shows an immunoblot of RE3 (fraction 14), RE4 (fraction 12), RE5 (fraction 17), and C2 (fractions 43 and 45) with anti-PV100 antibodies. Surprisingly, the polyclonal antibodies recognized C2 peptide efficiently, but no RE peptides at all appeared on the blot. These results suggested that the antigenicity of the Cys-rich peptides was much higher than that of the extremely hydrophilic Arg/Glu-rich peptides. On the immunoblot of protein storage vacuoles, the signal corresponding to <10 kDa might be caused by C2 peptide (Fig. 4*B*). The intensity of the signal was much higher than that of the 50-kDa vicilin-like protein. It seems likely that such CXXXC motifs cause allergy to animals as buckwheat trypsin inhibitor does (35).

VPE Mediates the Conversion of PV100 into Multiple Seed Proteins—We previously showed that VPE is involved in maturation of various seed proteins in the protein storage vacuoles by cleaving a peptide bond on the carbonyl side of Asn residues

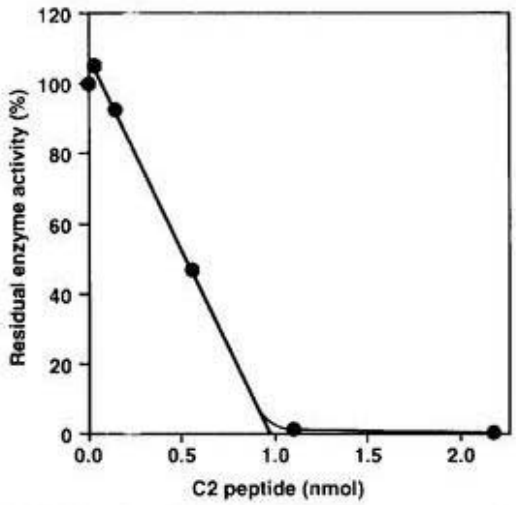


FIG. 7. **PV100-derived C2 peptide functions as a trypsin inhibitor.** The C2 peptide was highly purified from soluble fraction of the protein storage vacuoles of pumpkin seeds by HPLC. The reaction mixture contained 0–2.4 nmol of the C2 peptide, 10 μg of trypsin and 333 μg of BAPA in a 0.9-ml solution of 0.1 M Tris-HCl (pH 8.0) and 25 mM $CaCl_2$ (see under "Experimental Procedures"). The residual enzyme activity was monitored with absorbance at 405 nm.

(6, 10, 15). This raised the question of whether VPE mediates the proteolytic processing of PV100. To answer this question, we performed an *in vitro* processing of PV100 by the purified VPE from castor bean seeds. We used proproteins in the isolated PAC vesicles as substrates, including PV100, proglobulin, and pro2S albumin, as shown in Fig. 1*A* (*lane 1*). After incubation of these proteins with the purified VPE, the amount of PV100 decreased in association with the increase of the amount of a 50-kDa protein and <10-kDa proteins, as shown in Fig. 8. The <10-kDa proteins contained not only PV100-derived small proteins but also 2S albumin, composed of 3.8- and 8.0-kDa subunits, which was produced from pro2S albumin. This indicated that VPE was involved in the conversion of PV100 into the 50-kDa protein and the <10-kDa proteins.

The 50-kDa protein was subjected to automatic Edman degradation after digestion by pyroglutamate aminopeptidase (Fig. 8). The determined NH_2-terminal sequence, <QVAIR-

RTEQEQSNNPY, was found in the sequence of PV100, as in Fig. 2 (*arrow*). The NH_2-terminal sequence determined after *in vitro* processing was consistent with that of the 50-kDa protein accumulated in maturing seeds (data not shown). This suggests that processing similar to the *in vitro* processing of PV100 occurs by endogenous VPE during seed maturation. The result indicated that VPE mediated the production of the 50-kDa vicilin-like protein by cleaving an Asn^{375}-Gln^{376} bond of PV100. The cleavage was consistent with the substrate specificity of VPE toward Asn residues. Further degradation to remove NH_2-terminal three amino acids must occur at the later stage of seed maturation and produce a mature 50-kDa vicilin-like protein with the NH_2-terminal sequence, IRRTEQEQSNNPYYFQ (Figs. 2 and 4*B*). We previously reported that protein storage vacuoles accumulated not only VPE but also aspartic proteinase (36). It seems likely that such aspartic proteinase might be involved in the proteolytic trimming.

It should be noted that most processing occurs at Asn-Gln bonds in the hydrophilic region of PV100, and all of the mature proteins, the 50-kDa protein, and C2 and RE peptides have a pyroglutamate at their NH_2 termini, as shown in Fig. 9. Similar VPE-mediated processing of PV100 might occur to produce the multiple seed proteins (discussed below).

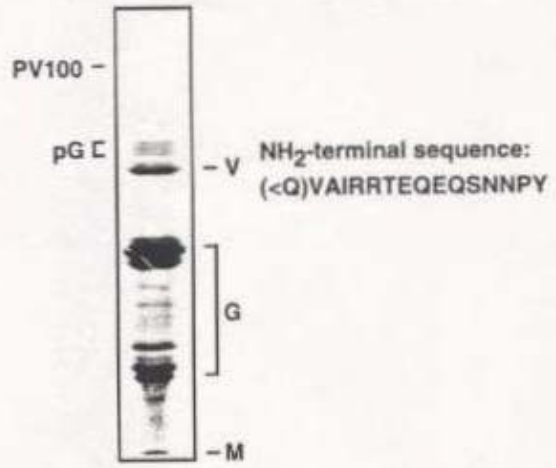


FIG. 8. ***In vitro* processing of PV100 by purified VPE produced the vicilin-like protein.** PAC vesicles that contained PV100 were incubated with the purified VPE and then subjected to SDS-PAGE followed by staining with Coomassie Blue. The resultant band corresponding to 50-kDa vicilin-like protein (*V*) that had been blotted to a polyvinylidene difluoride membrane was incubated with pyroglutamate aminopeptidase and then was subjected to automatic Edman degradation. The determined NH_2-terminal sequence corresponds to the sequence in PV100, as indicated by an *arrow* in Fig. 2. *pG* and *G* represent proglobulin and 11S globulin, respectively. The <10-kDa band (*M*) contained a mixture of the PV100-derived peptides and 2S albumin subunits that had been produced from pro2S albumin by VPE in the reaction.

DISCUSSION

PV100 Is a Unique Precursor to Multiple Functional Proteins—The present study demonstrates that PV100 is not only a precursor of vicilin storage protein but also a precursor of the Arg/Glu-rich RE peptides and a precursor of the Cys-rich C2 peptide that acts as a trypsin inhibitor. PV100 is a unique precursor for multiple seed proteins with different functions.

The C2 peptide was shown to have trypsin inhibitory activity. However, the sequence of the C2 peptide has no homology to known trypsin inhibitors, including members of squash trypsin inhibitor family (34), except for buckwheat trypsin inhibitor, showing a 18% identity to the C2 peptide. Interestingly, despite such low homology in primary structure, the higher structure of the C2 peptide might be analogous to that of buckwheat inhibitor. It has been shown that the buckwheat trypsin inhibitor forms a hairpin structure, in which two CXXXC motifs are linked by two disulfide bonds, and that Arg^{19}, between the two CXXXC motifs, is the reactive site for trypsin (35). Similarly, all four Cys residues of the C2 peptide formed two disulfide bonds, and Arg^{21} is found between the two CXXXC motifs (Fig. 6*B*). The result suggests that both the C2 peptide and buckwheat inhibitor belong to a novel family of trypsin inhibitors. These inhibitors might play a role in protecting the seeds from animals.

Among the Arg/Glu-rich RE peptides, the mature RE3 with the highest pI value (pI 11.90) shows the highest content in pumpkin seeds. We compared the RE3 composed of 36 amino acids with the pumpkin basic peptide that has been shown to be toxic to mouse B-16 cells (33) (Fig. 3*C*). The cytotoxic basic peptide was composed of 36 amino acids, and the probable amino acid sequence of the peptide was reported by Naisbitt *et al.* (33). Both sequences are identical to each other, except for two residues. It is likely that the mature RE3 accumulated in the vacuoles of pumpkin seeds might be identical to the cytotoxic basic peptide that was characterized by Naisbitt *et al.* (33). This suggests that the mature RE3 might function as a toxin to prevent animals from eating the seeds.

Most vacuolar proteins are synthesized as a proprotein precursor on the rough endoplasmic reticulum and are then transported to vacuoles. The vacuolar targeting signals have been shown to be present in the propeptides of some vacuolar proteins, including barley aleurain (37), barley lectin (38), sweet potato sporamin (39), and tobacco chitinase (40). It has been thought that the propeptides are cleaved off and degraded after

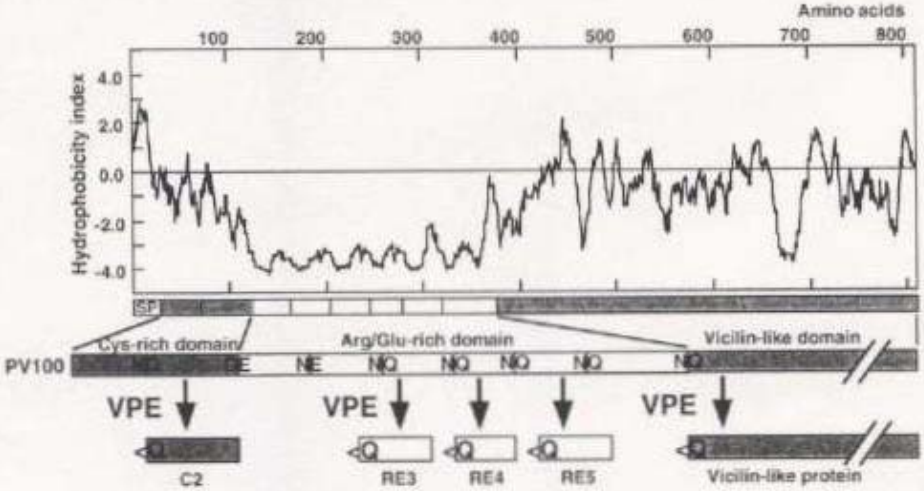


FIG. 9. **Hydropathy profile of PV100 and a hypothetical mechanism for the VPE-mediated cleavage at Asn-Gln bonds to produce multiple seed proteins.** The mean hydrophobicity index was computed according to the algorithm of Kyte and Doolittle (24) with a window of 10 residues. VPE is responsible for maturation of multiple seed proteins by cleaving Asn-Gln bonds that are found in the hydrophilic region of the PV100. Gln at the new NH_2 termini of the mature proteins might be spontaneously converted into pyroglutamate (<*Q*) under the acidic condition in the vacuoles. The cysteine-rich C2 peptide, the Arg/Glu-rich RE3, RE4, and RE5 peptides, and the vicilin-like protein are produced. *SP* represents a signal peptide.

arrival of the proproteins at the vacuoles. However, the possibility cannot be excluded that the propeptides exhibit some functions in the vacuoles after being removed from the precursor proteins, as the 4–6-kDa RE and C2 peptides are accumulated to act as functional proteins in the vacuoles.

VPE-mediated Cleavage at Asn-Gln Bonds of PV100 to Produce Multiple Seed Proteins with a Pyroglutamate at Their NH_2 Termini—Fig. 9 shows the hydrophobicity plot of PV100 and a hypothetical mechanism for vacuolar processing of PV100 to produce C2 peptide, RE peptides, and a vicilin-like protein. PV100 contains nine Asn-Gln bonds in the sequence. All six Asn-Gln bonds to be cleaved are located in the hydrophilic region of the PV100 sequence, whereas the other three noncleavable Asn-Gln bonds are found in the hydrophobic region of the vicilin-like domain. The result is consistent with our previous data showing that VPE recognizes Asn residues that are located in the hydrophilic region and are exposed on the surface of precursor molecules (6).

We previously reported that one subunit of pumpkin 11S globulin has a pyroglutamate at the NH_2 terminus (41). NH_2-terminal sequencing of the proglobulin in the isolated PAC vesicles revealed that a cleavage of Asn-Gln bond by VPE produced a pyroglutamate at the NH_2 terminus of the 11S globulin subunits (11). VPE cleavage of an Asn-Gln bond gives a new NH_2-terminal Gln residue, which might be spontaneously converted into a pyroglutamate under the acidic condition in the vacuoles. Proteins with an NH_2 terminus blocked by pyroglutamate are resistant to aminopeptidases that are localized in the vacuoles. These results suggested that the Asn-Gln sequences not only provide sites that can be cleaved by VPE but also produce aminopeptidase-resistant functional proteins in the vacuoles.

In contrast to the accumulation of RE3, RE4, RE5, and C2 peptide in the vacuoles, none of RE1, RE2, RE6, or C1 peptides were detected in the vacuoles (Fig. 6). They might be sensitive to proteinases in the vacuoles. The C1 peptide has two Asn residues inside the sequence (Fig. 2) and can be attacked by VPE to be degraded. It should be noted that the RE3, RE4, RE5, and C2 peptides have no Asn residue inside their sequences, indicating that they are resistant to VPE. They are also resistant to aminopeptidase because of a pyroglutamate at their NH_2 termini. However, both RE1 and RE2, with a Glu residue at each NH_2 terminus, could be sensitive to aminopeptidases, if they were produced by cleaving an Asp^{118}-Glu^{119} bond and an Asn^{161}-Glu^{162} bond by VPE, respectively.

Further proteolysis for COOH-terminal trimming at the Asp residues must occur to make the final mature forms of the RE3, RE4, and RE5 peptides (Fig. 6). Recently, we have found that VPE also cleaves a peptide bond at carbonyl side of Asp, although the activity toward Asp is less than that toward Asn (data not shown). The finding is consistent with the report that the VPE homolog of vetch has a substrate specificity toward both Asn and Asp residues (42). Therefore, it seems likely that the COOH-terminal trimming of RE3, RE4, and RE5 peptides is also mediated by VPE.

Acknowledgments—We thank Prof. Shigekata Yoshida of Nagoya University Experimental Farm for growing the pumpkin plants. We are grateful to L. M. Melgarejo of the National University of Colombia for help with cDNA cloning, to Y. Koumoto and J. Morita for their skillful technique on column chromatography, to Y. Makino for helpful support on mass spectrometry and peptide sequencing, and to C. Nanba for growing pumpkin and castor plants.

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The Plant Journal (1999) 19(1), 1–11

Vacuolar processing enzyme is up-regulated in the lytic vacuoles of vegetative tissues during senescence and under various stressed conditions

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Summary

Vacuolar processing enzyme (VPE) has been shown to be responsible for maturation of various seed proteins in protein-storage vacuoles. *Arabidopsis* has three VPE homologues; βVPE is specific to seeds and αVPE and γVPE are specific to vegetative organs. To investigate the activity of the vegetative VPE, we expressed the γVPE in a *pep4* strain of the yeast *Saccharomyces cerevisiae* and found that γVPE has the ability to cleave the peptide bond at the carbonyl side of asparagine residues. An immunocytochemical analysis revealed the specific localization of the γVPE in the lytic vacuoles of *Arabidopsis* leaves that had been treated with wounding. These findings indicate that γVPE functions in the lytic vacuoles as the βVPE does in the protein-storage vacuoles. The βVPE promoter was found to direct the expression of the β-glucuronidase reporter gene in seeds and the root tip of transgenic *Arabidopsis* plants. On the other hand, both the αVPE and γVPE promoters directed the expression in senescent tissues, but not in young intact tissues. The mRNA levels of both *αVPE* and *γVPE* were increased in the primary leaves during senescence in parallel with the increase of the mRNA level of a senescence-associated gene (*SAG2*). Treatment with wounding, ethylene and salicylic acid up-regulated the expression of *αVPE* and *γVPE*, while jasmonate slightly up-regulated the expression of *γVPE*. These gene expression patterns of the *VPEs* were associated with the accumulation of vacuolar proteins that are known to respond to these treatments. Taken together, the results suggest that vegetative VPE might regulate the activation of some functional proteins in the lytic vacuoles.

Received 12 January 1999; revised 16 April 1999; accepted 7 May 1999.
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Introduction

In higher plants, seed proteins are synthesized on the rough endoplasmic reticulum as proprotein precursors and then transported to protein-storage vacuoles via precursor-accumulating (PAC) vesicles (Hara-Nishimura *et al.*, 1998a). The precursors are proteolytically processed to make their respective mature forms in the vacuoles. We have found an enzyme responsible for maturation of various storage proteins in the protein-storage vacuoles of maturing seeds and have designated it vacuolar processing enzyme (VPE) (Hara-Nishimura *et al.*, 1991; Hara-Nishimura *et al.*, 1993a). Molecular characterization of VPE revealed that it has a larger precursor (proVPE) that has no activity, and that removal of the propeptides is necessary for activation (Hara-Nishimura *et al.*, 1993a). Recently we have shown that proVPE is converted into the active VPE by self-catalytic proteolysis (Hiraiwa *et al.*, 1997b; Hiraiwa *et al.*, 1999). This indicates that no other factor is necessary to produce active VPE and that VPE itself is a key enzyme in determining the final conformation of the seed proteins.

VPE is a novel cysteine proteinase with a substrate specificity towards asparagine residues (Hara-Nishimura, 1998). *In vitro* processing assays with the purified VPE have demonstrated that VPE is the authentic processing enzyme mediating maturation of several seed proteins of pumpkin and castor bean, including 2S albumins, 11S globulins, 7S globulin, ricin and *Ricinus communis* agglutinin (Hara-Nishimura and Nishimura, 1987; Hara-Nishimura *et al.*, 1991; Hiraiwa *et al.*, 1997a; Shimada *et al.*, 1994). The detailed mechanism of the vacuolar processing was investigated for 2S albumin, a major storage protein of pumpkin (Hara-Nishimura *et al.*, 1993b). VPE recognizes two exposed asparagine residues on the molecular surface of the pro2S albumin and then cleaves the peptide bonds at the carbonyl sides of the asparagine residues. Recently, we found that VPE cleaves Asn-Gln bonds of a single precursor to produce multiple functional proteins including a trypsin inhibitor, cytotoxic peptides and a vicilin-like storage protein (Yamada *et al.*, 1999). Most of the known seed proteins, including storage proteins, and lectins of different plants, including both monocots and dicots, are produced by proteolytic cleavages at the asparagine residues that are found in a hydrophilic region of the precursor sequences, as reviewed by Hara-Nishimura *et al.* (1995). Therefore, VPE homologues could be

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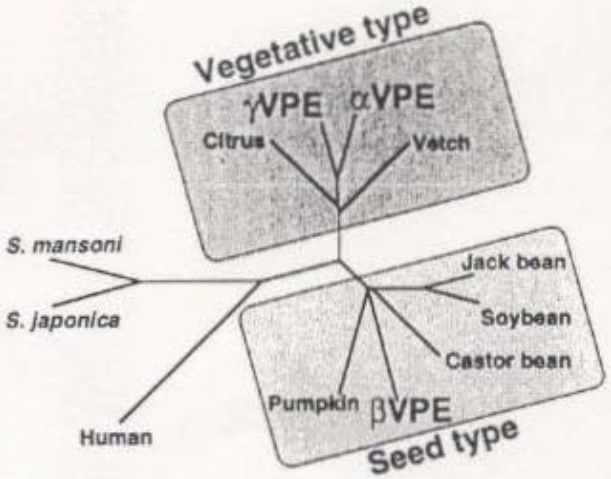

Figure 1. A phylogenetic tree of VPE homologues.
VPE homologues of a novel family of cysteine proteinases are distributed in higher plants and animals. Plant homologues can be separated into two types, a seed type and a vegetative type. The computer program CLUSTAL V (Higins *et al.*, 1992) was used to calculate the relative lengths of branches. References to the published sequences are as follows: *Arabidopsis* αVPE and βVPE (Kinoshita *et al.*, 1995a); *Arabidopsis* γVPE (Kinoshita *et al.*, 1995b); castor bean (Hara-Nishimura *et al.*, 1993a); soybean (Shimada *et al.*, 1994); pumpkin (unpublished data); jack bean (Takeda *et al.*, 1994); vetch (Becker *et al.*, 1995); citrus (Alonso and Granell, 1995); human (Chen *et al.*, 1997), *Schistosoma mansoni* (Klinkert *et al.*, 1989) and *Schistosoma japonicum* (accession number S31908).

distributed in a wide range of plant species to mediate the maturation of these seed proteins.

Four cDNAs were isolated from maturing seeds of castor bean (Hara-Nishimura *et al.*, 1993a), soybean (Shimada *et al.*, 1994), pumpkin (unpublished data), and Jack bean (Takeda *et al.*, 1994) and two cDNAs for VPE homologues were isolated from vegetative organs, such as germinating cotyledons of vetch (Becker *et al.*, 1995) and ripening citrus fruits (Alonso and Granell, 1995). We have isolated three genomic clones for *Arabidopsis* VPEs, αVPE, βVPE and γVPE (Kinoshita *et al.*, 1995a; Kinoshita *et al.*, 1995b). These homologues belong to a novel family of cysteine proteinases and the plant homologues can be separated into two subfamilies: one specific to seeds and the other specific to vegetative organs, as shown in Figure 1. This is consistent with the fact that the plant vacuoles are classified into two types, protein-storage vacuoles and lytic vacuoles.

These classifications of both plant vacuoles and VPEs imply that αVPE and γVPE might play a role in the maturation of some proteins in the lytic vacuoles of the vegetative organs of *Arabidopsis*, whilst βVPE might be involved in the maturation of the seed proteins in the protein-storage vacuoles of maturing *Arabidopsis* seeds. The VPE-mediated maturation system for seed proteins has been well characterized, as described above. A similar VPE-mediated system could be involved in maturation of vacuolar proteins in the vegetative vacuoles to acquire and maintain their active functions of vacuoles. To address this possibility, it is necessary to clarify when and where the vegetative VPEs are expressed in order for them to function in the lytic vacuoles. Here we demonstrate the enzyme activity and subcellular localization of vegetative γVPE of *Arabidopsis*, and show the temporal and spatial expression of the vegetative *VPE* genes. We also show that the expression patterns of the vegetative *VPE* genes in senescent tissues are associated with the accumulation of hydrolytic enzymes to be converted into their mature forms by VPE.

Results

Vegetative γVPE of Arabidopsis *has a vacuolar processing activity*

Previously we detected the vacuolar processing activity in the vegetative tissues, including mature leaves, roots and hypocotyls of castor bean, pumpkin and mung bean, although the specific activity was much lower than that of the seeds of these plants (Hiraiwa *et al.*, 1993). To investigate the processing activity of the vegetative γVPE of *Arabidopsis*, we expressed it in a *pep4* strain of the yeast *Saccharomyces cerevisiae*. The extract from the transformant cells showed an ability to cleave the peptide bond at the carbonyl side of the asparagine residues of the synthetic peptide, as shown in Figure 2(a) (left). The sequence of the peptide was derived from the sequence around the processing site of proglobulin, the proprotein precursor of 11S globulin of pumpkin (Hayashi *et al.*, 1988). The cleavage was inhibited by the addition of 1 mM *N*-ethylmaleimide (data not shown). This is consistent with the fact that VPEs are cysteine proteinases (Hara-Nishimura, 1998). The extract from the transformant with the vector alone showed no activity (Figure 2a, right).

The γVPE also showed the processing activity on a proprotein precursor, yeast carboxypeptidase Y (CPY). It has been shown that procarboxypeptidase Y (proCPY) is processed at the peptide bond of the carbonyl side of asparagine-111 (Valls *et al.*, 1987) and the maturation of CPY is dependent on proteinase A, a *PEP4* gene product (Jones *et al.*, 1982). Previously, we found that the seed VPE recognized the asparagine residue to be cleaved and mediated the maturation of CPY instead of proteinase A (Hiraiwa *et al.*, 1997b). Figure 2(b) shows that the vegetative γVPE was also transported into the yeast vacuoles and was involved in the maturation of CPY in the proteinase A-deficient (*pep4*) strain. These results indicate that the vegetative γVPE has a vacuolar processing activity, as do seed VPEs.

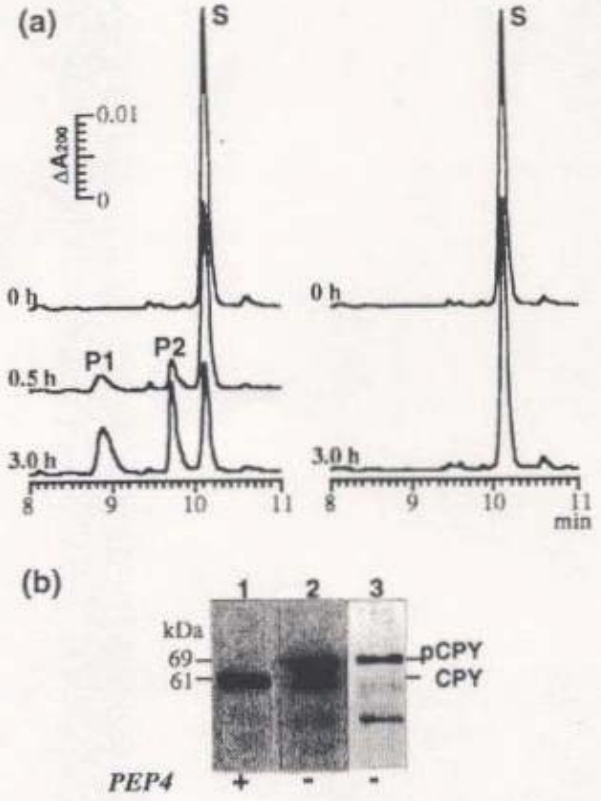

Figure 2. Vacuolar processing enzyme activity of the vegetative γVPE.
(a) The processing enzyme activity was assayed with a synthetic decapeptide (S) as substrate (see text for details). Vegetative γVPE was expressed in yeast cells and was used for the assay (left). The products (P1 and P2) of the reaction for 0, 0.5 and 3.0 h were analyzed by capillary electrophoresis. The extract from the yeast cells transformed with a vector alone was used for the assay (right).
(b) *In vivo* processing assay on a proprotein precursor was performed by monitoring the processing of procarboxypeptidase Y (pCPY) into the mature form (CPY) (see text for details). Galactose-grown transformant cells were subjected to SDS-PAGE and subsequent immunoblot analysis with CPY-specific antibodies. Wild-type (*PEP4*) cells (lane 1) and the proteinase A-deficient (*pep4*) cells expressing γVPE (lane 2) and the proteinase A-deficient cells transformed with a vector pYES2 alone (lane 3) were exsmined.

Vegetative γVPE is specifically localized in the lytic vacuoles of the Arabidopsis *leaves*

The next issues to be resolved were whether the vegetative VPEs are synthesized in the vegetative tissues of plants and, if they are, where is their subcellular localization? To answer these questions, we performed an immunocytochemical analysis with specific antibodies against γVPE. We used the 5th leaves of 21-days-old *Arabidopsis* plants that had been incubated for 48 h after wound treatment (see Figure 7a). Immunoelectron microscopy of the tissues only revealed gold particles in electron-dense structures in the lytic vacuoles of the leaves, as shown in Figure 3. We have statistically analyzed

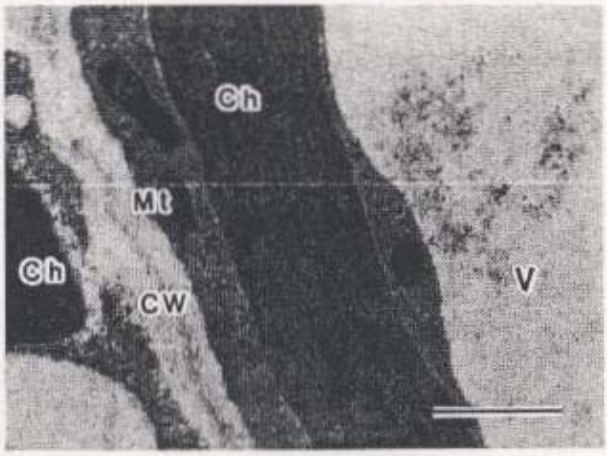

Figure 3. Immunocytochemical localization of the vegetative γVPE in *Arabidopsis* leaves.
An ultra-thin section of one of the 5th leaves of a 21-days-old *Arabidopsis* plant was incubated with anti-γVPE antibodies and then protein A-gold. Ch, chloroplast; CW, cell wall; Mt, mitochondrion; V, vacuole. Bars = 1 mm.

the immunoelectron micrographs with either the specific antibodies or the pre-immune serum (data not shown). This result indicates clearly that γVPE is localized in the lytic vacuoles of the *Arabidopsis* leaves.

Differential expression of three VPE *genes in the seeds and seedlings of* Arabidopsis

Arabidopsis VPEs, αVPE, βVPE and γVPE, are divided into two classes on the phylogenetic tree (vegetative types and seed types), as are all VPE homologues that have been registered in the databases (Figure 1). To study the temporal and spatial expression of these *Arabidopsis* genes, we constructed three chimeric fusion genes composed of the promoter region of each *VPE* gene and the coding region of the β-glucuronidase (*gus*) gene and then introduced it to *Arabidopsis* plants. Figure 4 shows histochemical staining of GUS activity in seeds and 3-, 6-, 9- and 17-days-old plants of the transgenic *Arabidopsis* with each of three fusion genes: the *pαVPE::gus* gene, the *pβVPE::gus* gene and the *pγVPE::gus* gene.

Dry seeds of the transgenic plants with the *pβVPE::gus* gene show a high GUS activity (Figure 4d), while those of the plants with the *pαVPE::gus* gene or the *pγVPE::gus* gene show no activity (Figure 4a,g). This result is consistent with the classification of three *Arabidopsis* VPEs in the phylogenetic tree of Figure 1. The *pβVPE::gus* gene was expressed throughout the seeds including both the embryo axis and the cotyledons (Figure 4d). The expression in the seeds is in good agreement with the gene expression of a storage protein, 2S albumin (Guerche

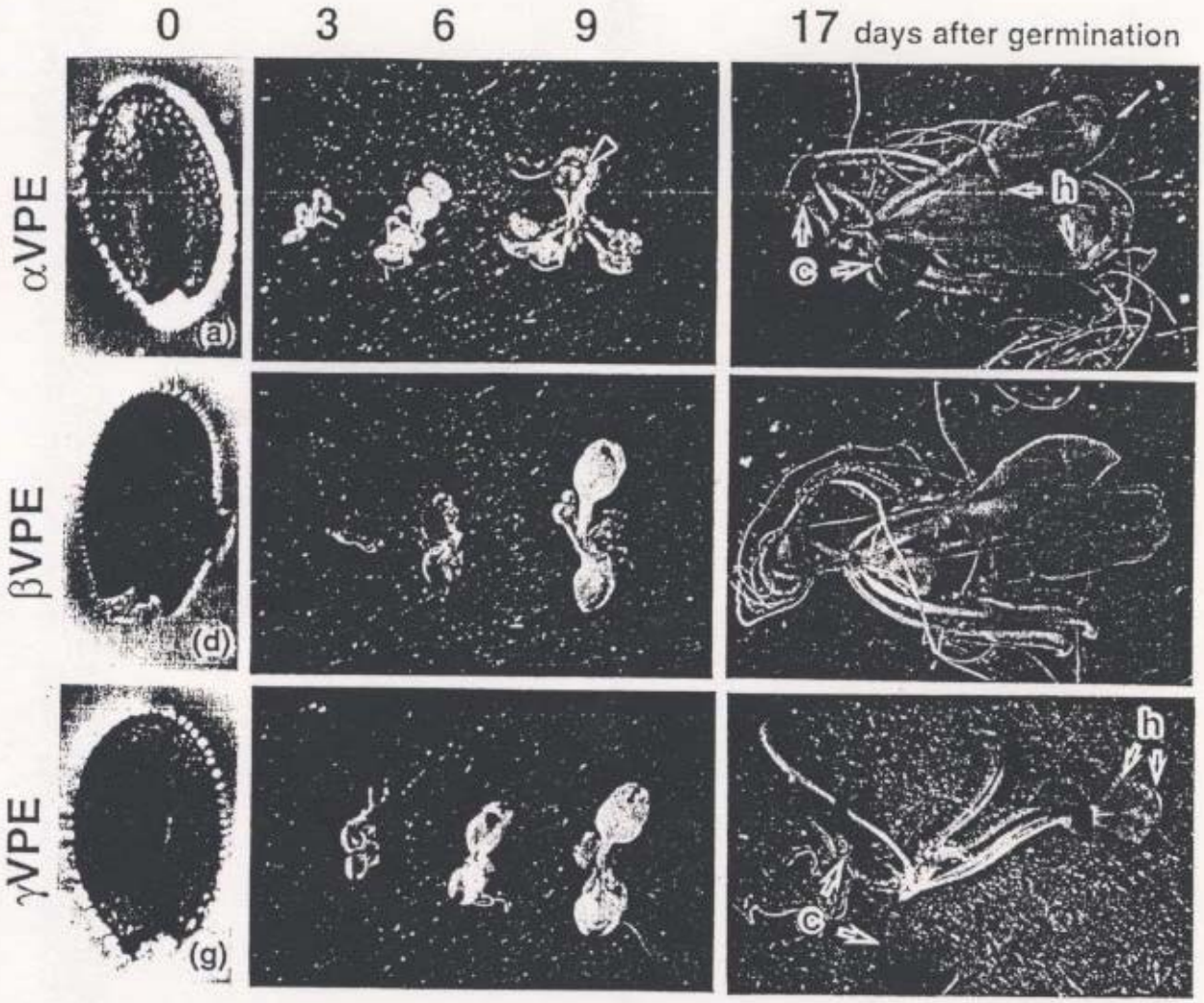

Figure 4. Differential expression of three *VPE* genes in seeds and seedlings of transgenic *Arabidopsis* plants.
Histochemical localization of GUS activity was examined with transgenic plants.
(a) A dry seed with the *pαVPE::gus* gene. (b) Three-, 6- and 9-days-old seedlings with the *pαVPE::gus* gene. An arrowhead indicates the vascular tissues stained blue. (c) A 17-days-old plantlet with the *pαVPE::gus* gene. (d) A dry seed with the *pβVPE::gus* gene. (e) Three-, 6- and 9-days-old seedlings with the *pβVPE::gus* gene. (f) A 17-days-old plantlet with the *pβVPE::gus* gene. (g) A dry seed with the *pγVPE::gus* gene. (h) Three-, 6- and 9-days-old seedlings with the *pγVPE::gus* gene. (i) A 17-days-old plantlet with the *pγVPE::gus* gene. c, cotyledon; h, hydathode.

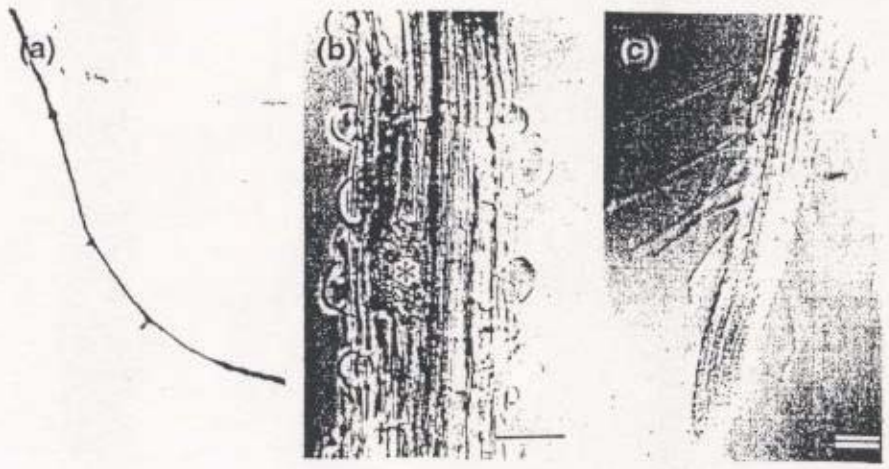

Figure 5. Differential expression of the *αVPE* gene in the roots of transgenic *Arabidopsis* plants.
Histochemical localization of GUS activity in sections of the roots was examined with a DIC or light microscope.
(a) A root with the *pαVPE::gus* gene. Branching points for lateral root formation are stained.
(b) A DIC micrograph showing one of the branching points shown in (a). Like the vascular tissue, cortex cells adjacent to an emerging lateral root (indicated by an asterisk) are stained.
(c) A DIC micrograph of a root with the *pαVPE::gus* gene. One of the vascular tissue is stained. Bars = 100 mm.

et al., 1990). GUS activity in the cotyledons with the *pβVPE::gus* gene was reduced during seedling growth until no activity was detectable in 17-days-old plants (Figure 4e,f).

In contrast to the plants with the *pβVPE::gus* gene, both the plants with the *pαVPE::gus* gene and those with the *pγVPE::gus* gene showed GUS activity in the old cotyledons and the rosette leaves of the 17-days-old plants (Figure 4c,i), but not in the dry seeds or the young seedlings (Figure 4a,b,g,h). The senescent tissues of 17-day-old plants, in particular, were strongly stained blue, although the stainability was much higher in the senescent cotyledons with the *pγVPE::gus* gene than in those with the *pαVPE::gus* gene (Figure 4c,i). It should be noted that the vegetative VPEs are not expressed throughout the vegetative organs, but in the tissues in which senescence is occurring.

The vegetative αVPE gene is expressed in root tissues in which cell differentiation is occurring

Figure 5(a–c) shows the histochemical staining of GUS activity in the roots of the transgenic *Arabidopsis* with the *pαVPE::gus* gene. GUS activity was detected at the branching points of the root of the transgenic plants (Figure 5a). An enlarged micrograph of one of the branching points shows that the cortex cells next to an emerging lateral root (indicated by an asterisk) were stained as well as the vascular tissue (Figure 5b). Programmed cell death has been detected in the cortex cells around emerging lateral roots of soybean by TUNEL staining (Kosslak *et al.*, 1997). It seems likely that αVPE might function in the lytic vacuoles of the root tissues in which cell death is occurring. Another case of αVPE gene expression related to cell death is shown in Figure 5(b,c). GUS activity was also detected in the developing vascular tissue of the roots, as well as the vascular tissue of the rosette leaves (Figure 4b, indicated by an arrowhead). Vascular tissues contain tracheary elements composed of dead cells that are formed via programmed cell death (Fukuda, 1992). The GUS staining in the vascular tissue and the cortex cells adjacent to the emerging lateral roots was specific to the *αVPE* gene. Only the *αVPE* gene was expressed in these tissues, in contrast to the expression of both *αVPE* and *γVPE* genes in the senescent leaves.

Vegetative VPE *mRNAs are accumulated in the leaves in association with senescence*

To examine the expression of the *αVPE* and *γVPE* genes in senescent tissues in greater detail, we performed a Northern blot analysis with the primary and secondary rosette leaves that were harvested from 15-, 20-, 25-, 30- and 35-days-old *Arabidopsis* plants. Figure 6(a) shows that the level of *γVPE* mRNA increased rapidly from 15 to 25 days after germination and that the level of *αVPE* mRNA increased at a later stage, from 25 to 35 days after germination. These increases were almost parallel with the decrease of chlorophyll content of the leaves (Figure 6b) and with the decrease of the content of Rubisco mRNA (data not shown). Interestingly, *γVPE* mRNA was detectable in green leaves just before starting senescence and *αVPE* mRNA was detectable only in senescent yellow leaves. This result was consistent with the histochemical staining of GUS activity in senescent leaves (Hara-Nishimura *et al.*, 1998b).

We also examined the developmental change in the mRNA levels of *SAG2* (senescence-associated gene; Hensel *et al.*, 1993) and *RD21* (dehydration-responsive gene; Koizumi *et al.*, 1993). Both the *SAG2* and *RD21* genes of *Arabidopsis* encode cysteine proteinases of the papain family. Figure 6(a) shows that the mRNA level of *γVPE* increased much more dramatically than did the mRNA levels of both SAG2 and RD21. The relationship between these inducible proteinases and vegetative VPEs is discussed below. No *βVPE* mRNA was detectable in the leaves as expected from the result shown in Figure 4(f).

Vegetative VPE *mRNAs are up-regulated in leaves by wounding, ethylene and salicylic acid, and slightly by jasmonate*

Various vacuolar proteins have been shown to be accumulated in the vacuoles in response to wounding, ethylene, salicylic acid and methyl jasmonate. This raises the question of whether the vegetative *VPE* genes are induced by such treatments. To answer the question, we performed an RNA blot analysis with the leaves of 21-days-old plants after various treatments. The plants were aseptically cultured on solid MS medium to avoid environmental stimuli, and the 5th and 6th leaves were used for extraction of total RNA.

To determine the effect of wound treatment on the expression of the *VPE* gene, the 5th and 6th leaves were mechanically wounded and then harvested 0, 6, 12, 24 and 48 h after wounding. Figure 7(a) shows that the level of *γVPE* mRNA in the leaves increased during the period 12–48 h after the wound treatment. This indicates that the *γVPE* gene is up-regulated in the tissues by wounding. The increase of *γVPE* mRNA preceded that of *αVPE* mRNA, which was first observed 24 h after the wound treatment. The amount of the induced *αVPE* mRNA was much lower than that of *γVPE* mRNA. Thus, it seems likely that the induction of *αVPE* mRNA might be caused by a secondary effect rather than directly by wounding.

In the next step, we examined the effects of treatment with ethylene and salicylic acid. Both *αVPE* and *γVPE*

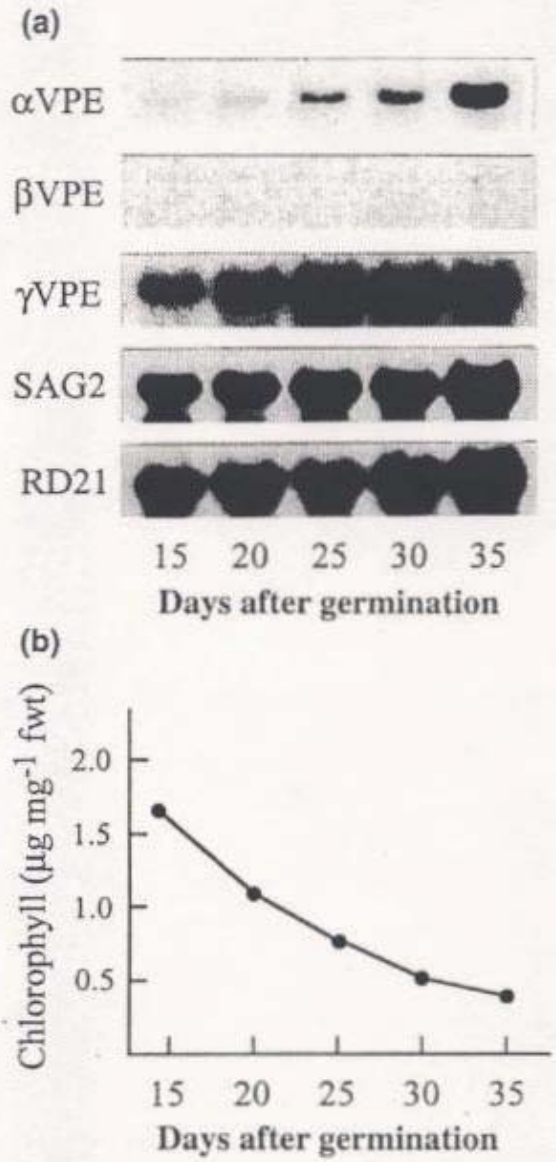

Figure 6. Vegetative *VPE* mRNAs are accumulated in the rosette leaves of *Arabidopsis* in association with senescence.
(a) Total RNA was extracted from the primary and secondary rosette leaves of 15-, 20-, 25-, 30- and 35-days-old plants of *Arabidopsis*. A Northern blot was performed with a single probe that was specific to *αVPE*, *βVPE*, *γVPE*, *SAG2* and *RD21*.
(b) Developmental changes in the chlorophyll content in the primary and secondary rosette leaves during senescence of the *Arabidopsis* plants.

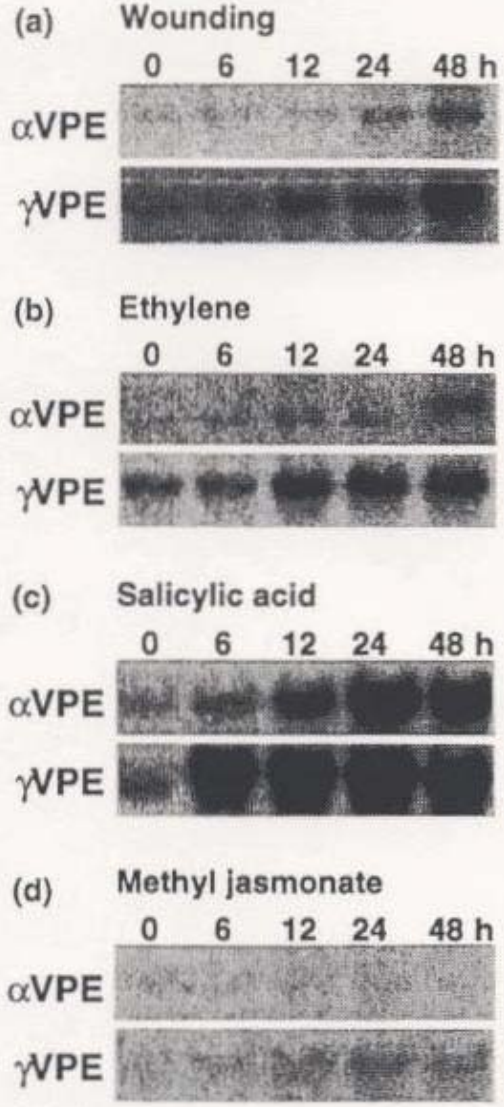

Figure 7. Vegetative *VPE* mRNAs are up-regulated in rosette leaves of *Arabidopsis* by wound treatment, ethylene and salicylic acid, and slightly by jasmonate.
The 5th and 6th rosette leaves were harvested from 21-days-old plants of *Arabidopsis* after 0, 6, 12, 24 and 48 h of each treatment. Total RNA was extracted from the leaves for a Northern blot with a single probe specific to *αVPE* and *γVPE*.
(a) The leaves were mechanically wounded with tweezers.
(b) The plants were treated with 100 μl L^{-1} ethylene gas.
(c) The plants were sprayed with 2.5 mM salicylic acid.
(d) The plants were sprayed with 60 μM methyl jasmonate.

mRNAs are expressed in response to ethylene and salicylic acid, as shown in Figure 7(b,c). However, salicylic acid caused a much stronger induction of both mRNAs than ethylene did. The most rapid response of the *γVPE* gene occurred 6 h after the salicylic acid treatment (Figure 7c). Finally, methyl jasmonate showed no effect on the gene expression of *αVPE* and a slight induction of *γVPE* gene, as shown in Figure 7(d).

Discussion

βVPE might function in protein-storage vacuoles, while both αVPE and γVPE might function in lytic vacuoles

Seed VPEs of castor bean and pumpkin have been shown to be localized in protein-storage vacuoles (Hara-Nishimura and Nishimura, 1987; Hara-Nishimura *et al.*,

1993a; Hiraiwa *et al.*, 1993) and to function as key enzymes responsible for maturation of various seed proteins (Hara-Nishimura and Nishimura, 1987; Hara-Nishimura *et al.*, 1991; Hara-Nishimura *et al.*, 1995). In castor bean, both the activity and protein levels of the VPE rapidly decrease in the endosperm during seed germination (Hiraiwa *et al.*, 1993). This result is consistent with the decrease of *βVPE* gene expression in the *Arabidopsis* cotyledons after seed germination (Figure 4e). These results indicate that *Arabidopsis* βVPE is a counterpart of the seed VPE of castor bean and plays a role in maturation of seed proteins in protein-storage vacuoles of maturing *Arabidopsis* seeds.

On the other hand, both *αVPE* and *γVPE* mRNAs are expressed in the vegetative organs of *Arabidopsis*. The vegetative γVPE with the vacuolar processing activity was shown to be localized in the lytic vacuoles of the leaves in this study (Figures 2 and 3), an indication that the *γVPE* gene product is the authentic VPE in the lytic vacuoles of *Arabidopsis*. This result supports the possibility that a VPE-mediated system similar to that in seeds might function in the lytic vacuoles.

Two VPE homologues were purified from germinating cotyledons of vetch (proteinase B; Becker *et al.*, 1995) and *Vigna mungo* (VmPE-1; Okamoto and Minamikawa, 1995). These enzymes have a substrate specificity towards asparagine residues. The level of mRNA of proteinase B in the cotyledons increases during seed germination (Becker *et al.*, 1995). This is in good agreement with the gene expression of the *αVPE* and *γVPE* genes in the cotyledons (Figure 4c,i). Taken together, these results suggest that VPE systems are widely distributed both in protein-storage vacuoles and in lytic vacuoles of various plant tissues.

Senescence-inducible cysteine proteinase as a putative target protein of vegetative VPE in senescent tissues

The results mentioned above raise the question of what are the target proteins of the vegetative VPEs? The histochemical localization of GUS activity in transgenic plants and the RNA blot analysis revealed that vegetative VPE genes are not expressed in healthy leaves, but are expressed in senescent leaves (Figure 4). This implies that the target proteins of vegetative VPEs might also be senescence-inducible hydrolytic enzymes localized in the vacuoles. Figure 6 indicates that two cysteine proteinases of *Arabidopsis*, SAG2 and RD21, are induced during the senescence of the leaves in association with induction of the vegetative VPEs.

Figure 8a shows various cysteine proteinases that appeared in tissues undergoing senescence and degeneration of different plants. These include actinidin in ripening kiwi fruits (Lin *et al.*, 1993), C14 in ripening tomato (Drake *et al.*, 1996), Cp in senescent carnation and daylily flowers (Jones *et al.*, 1995; Valpuesta *et al.*, 1995), Top in senescent pea ovaries (Granell *et al.*, 1992), oryzain α and oryzain β in degenerating endosperm of rice (Watanabe *et al.*, 1991) and RD21 in drought-stressed *Arabidopsis* leaves (Koizumi *et al.*, 1993). These proteinases are members of the papain family. We have revealed that vegetative *VPE* genes were expressed in the ripening fruit of transgenic tobacco plants, as well as in their senescent tissues (unpublished data). The inactive precursor of kiwi fruit actinidin, one of the better characterized proteinases, has been shown to be converted into an active enzyme by the removal of a C-terminal propeptide at Asn-Gln (Baker, 1980; Paul *et al.*, 1995). The cleavable asparagine residue is conserved in a hydrophilic region of the precursor polypeptides of all the cysteine proteinases shown in Figure 8(a). It seems likely that the vegetative VPEs with a substrate specificity towards asparagine residues might be involved in maturation and activation of the inducible cysteine proteinases in the lytic vacuoles of senescing and degenerating tissues. This is also supported by the evidence that a VPE homologue, VmPE-1, is involved in post-translational processing of a cysteine endopeptidase SH-EP to activate the enzyme in germinating cotyledons of *Vigna mungo* (Okamoto and Minamikawa, 1995).

A pathogen-related protein, chitinase and wound-inducible proteinase inhibitors are putative target proteins of vegetative VPE in damaged tissues

It has been shown that proteinase inhibitors of tomato leaves (Graham *et al.*, 1985) and tobacco stigmas (Atkinson *et al.*, 1993) are induced in the vacuoles by wound treatment. The inhibitors are synthesized as proprotein precursors and then converted into the mature inhibitors by post-translational cleavages at specific asparagine residues (Atkinson *et al.*, 1993; Graham *et al.*, 1985), as shown in Figure 8(b). Vegetative *VPE* mRNAs were up-regulated in response to mechanical wounding (Figure 7a). The wound-inducible VPEs could mediate the maturation of the inhibitors by cleaving peptide bonds at the asparagine residues.

When pathogens attack plants, salicylic acid and ethylene mediate the accumulation of defensive proteins (PR proteins) in the vacuoles and the extracellular matrix. A basic chitinase of tobacco leaves, one of the PR proteins that accumulates in the vacuoles, has been shown to be converted from the proprotein precursor by the removal of a C-terminal propeptide at an asparagine residue (Sticher *et al.*, 1993), as shown in Figure 8(b). Treatment of the leaves with salicylic acid and ethylene also up-regulated vegetative *VPE* mRNAs (Figure 7b,c). The vegetative VPEs

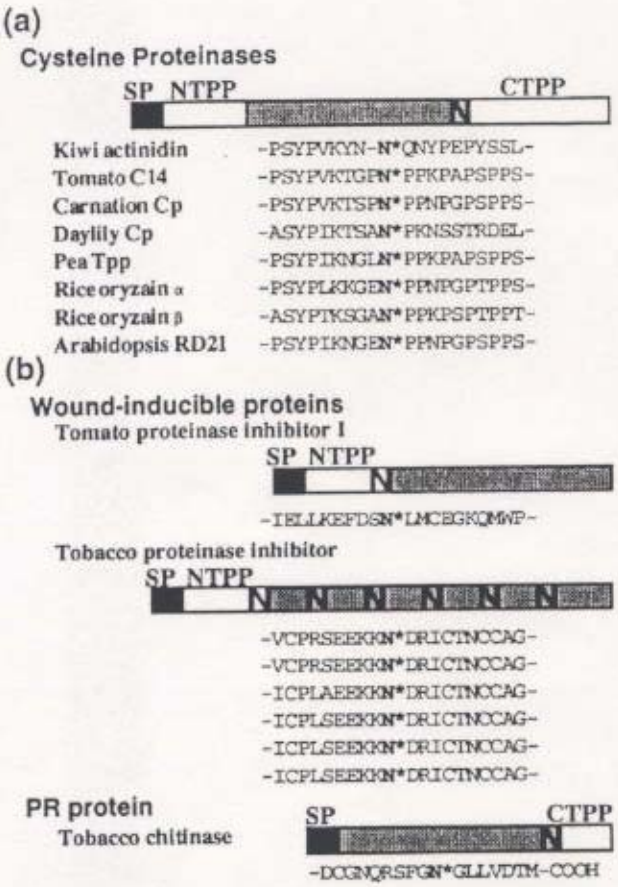

Figure 8. Putative target proteins for vegetative VPEs in lytic vacuoles.
The sequences around proteolytic processing site of precursors of putative target proteins for VPEs. The processing occurs on the C-terminal side of an asparagine residue that is indicated by an asterisk. SP, a signal peptide; NTPP, N-terminal propeptide; CTPP, C-terminal propeptide; N, an asparagine residue.
(a) Various cysteine proteinases of the papain family are induced during senescence of fruit (Drake *et al.*, 1996; Lin *et al.*, 1993), flowers (Jones *et al.*, 1995; Valpuesta *et al.*, 1995), ovary (Granell *et al.*, 1992), endosperm (Watanabe *et al.*, 1991) and during drought stress of leaves (Koizumi *et al.*, 1993). It has been shown that in order to activate actinidin and oryzain-α, a post-translational cleavage at an asparagine residue is needed to remove a C-terminal propeptide. Asparagine residues at the processing site are conserved among these proteinase precursors.
(b) Proteinase inhibitors of tomato (Graham *et al.*, 1985) and tobacco (Atkinson *et al.*, 1993) have been shown to be induced in the lytic vacuoles by wound treatment. Tobacco chitinase is a basic PR protein accumulated in the lytic vacuoles during a hypersensitive reaction of the tissue. Proprotein precursors of these proteins are post-translationally processed at asparagine residues to produce the mature inhibitors and a chitinase.

up-regulated in response to ethylene could be involved in the maturation of the chitinase to defend the tissues against pathogens.

These treatments may have stressed the cells, in which newly synthesized proteins would have been accumulated in the vacuoles. Ethylene and salicylic acid treatments have been shown to induce different defensive proteins, called pathogenesis-related proteins (PR proteins), which are accumulated in the vacuoles or the extracellular space to defend the cells against attacks from pathogens (Bol and Linthorst, 1990). Taken together, the present results suggest that the vegetative VPEs might regulate activation of such functional proteins to give the lytic vacuoles new functions, including defense of the cells.

Vegetative VPEs could be useful markers of the vegetative vacuoles in cells that are actively differentiating

The lower expression of the vegetative *VPE* genes in young leaves revealed that VPE function might not be associated with vacuolar enlargement. In contrast, high expression of these *VPE* genes was observed in senescent and stressed tissues, in which the lytic vacuoles have been shown to accumulate newly synthesized proteins to be converted into mature forms and to degrade disused cellular components. The high expression of vegetative VPEs could reflect the active accumulation of functional proteins in the vacuoles and the rapid degradation of unnecessary proteins to differentiate the cells. Thus, the VPEs could be a good marker to identify functionally active vacuoles to be differentiated in the tissues.

The GUS staining in the vascular tissue and the cortex cells adjacent to the emerging lateral roots was specific to the *αVPE* gene (Figure 5). Only the *αVPE* gene was expressed in these tissues, in contrast to the expression of both *αVPE* and *γVPE* genes in the senescent leaves and the leaves treated with wounding, ethylene and salicylic acid. Further analysis of each functional site of *αVPE* and *γVPE* will be required to clarify the distinct role of each VPE in tissues that are undergoing senescence and in those that are under stress.

Experimental procedures

Growth conditions of Arabidopsis *plants*

Seeds of *Arabidopsis thaliana* ecotype Columbia were surface-sterilized with a solution of 2% sodium hypochlorite and 0.01% Triton X100 and then sown onto 0.5% Gellan Gum (Wako, Tokyo, Japan) that contained MS medium, 100 mg l^{-1} myo-inositol and 2.5 mM MES-KOH (pH 5.7). After a 2-day incubation at 4°C to break seed dormancy, the seeds were germinated and grown for 35 days at 22°C under continuous light (100 mE s^{-1} m^{-2}).

The primary and secondary rosette leaves that were harvested from the 15-, 20-, 25-, 30- and 35-days-old plants that were aseptically grown in the plastic pots to eliminate the environmental stimuli were used for extraction of mRNAs. For the experiments to study the response to ethylene gas and other treatments, 21-days-old plants were used. The plants were transferred to an airtight box containing 100 ml l^{-1} of ethylene gas or were sprayed with 2.5 mM salicylic acid solution or 60 mM methyl jasmonate. For wound treatment, the 5th and 6th rosette leaves of each 21-days-old plant were mechanically wounded at

two sites per leaf with tweezers. The wounded leaves were harvested from the plants that had been incubated for 0, 6, 12, 24 or 48 h after the treatment.

RNA gel blot analysis and measurement of chlorophyll content

Total RNA was extracted from the leaves with a commercial kit (ISOGEN; Nippon Gene, Tokyo, Japan) and precipitated with LiCl for further purification. Total RNA (10 μg) denatured with formamide and formaldehyde was separated on a 1% agarose gel containing formaldehyde and stained with ethidium bromide to ensure that equal amounts of RNA had been applied to the different lanes in the gel. After electrophoresis, the RNA was transferred to a nylon membrane (Hybond N+; Amersham Japan, Tokyo). For probes that were specific to each of the *VPE* genes, we used amplified DNAs of about 270 bp that contained either the 6th exon of the *αVPE* gene, the 7th exon of the *βVPE* gene or the 7th exon of the *γVPE* gene. These sequences are located in the most diverse region among the three *VPE* genes. The amplified DNA fragments were labeled with [^{32}P]dCTP by a random priming method. Pre-hybridization and hybridization were performed as described previously (Kinoshita *et al.*, 1995a). We used two EST clones as DNA probes; SAG2 (116M8T7) and RD21 (103I15T7) from the Arabidopsis Biological Research Center.

The chlorophyll content was spectrophotometrically measured as described previously (Arnon, 1949).

Construction of chimeric gene of a VPE *promoter-*gus *gene and transformation of* Arabidopsis

We used three genomic clones for *αVPE*, *βVPE* and *γVPE* that had been previously isolated (Kinoshita *et al.*, 1995a; Kinoshita *et al.*, 1995b). DNA fragments of the 5′-upstream regions from −1651 to +5 of the *αVPE* gene, from −2286 to +11 of the *βVPE* gene and from −2036 to +11 of the *γVPE* gene were amplified by PCR. The following primers were synthesized to amplify each promoter region; 5′-GGAAGCTTATCCCAACACAAGACATAA-3′ and 5′-TACCCGGGGTCATTGTTAGGTGGTATTCTC-3′ for *αVPE*, 5′-CCAAGCTTAAACTCCAGCTTACTTGTAT-3′ and 5′-TACCCGGGAGACTTAGCCATGGCAGCGA-3′ for *βVPE* and 5′-CCAAGCTTCCGGTTTCATTATGGTTAAG-3′ and 5′-AACCCGGGACACGTGTCATCGTTGTGG-3′ for *γVPE*. A *Hin*dIII site was incorporated at the distal end of the sense primers and an *Sma*I site was incorporated at the proximal end of the sense primers. The amplified fragments were digested with *Hin*dIII and *Sma*I, and then incorporated into a binary vector, pBI121HmRV, to fuse with the β-glucuronidase (*gus*) gene. pBI121HmRV, a derivative of pBI121, contains a hygromycin phosphotransferase gene at a unique *Eco*RI site in the region downstream of the *gus* gene in the reverse orientation. *Agrobacterium tumefaciens* EHA101 cells were transformed with these plasmids containing *αVPE* promoter-*gus* (*pαVPE::gus*), *βVPE* promoter-*gus* (*pβVPE::gus*) and *γVPE* promoter-*gus* (*pγVPE::gus*), respectively, by the electroporation method (Nagel *et al.*, 1990). *Arabidopsis* plants were transformed using a tissue culture method (Akama *et al.*, 1992) and the *in planta* method (Bechtold *et al.*, 1993).

Histochemical detection of β-glucuronidase activity

Tissues from the transgenic plants were incubated for 1–18 h in a substrate solution containing 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc, 0.5 mg ml^{-1}), 0.1% (w/v) Triton X-100, 100 mM sodium phosphate (pH 7.4), as reported (Jefferson, 1987). To stop the reaction and to remove the chlorophyll, the tissues were treated with an ethanol series. All experiments for analysis of GUS activity were repeated at least three times using nine independent lines of the transgenic plants with each of the chimeric genes.

Heterologous expression of γVPE in yeast cells

The entire reading frame of the γVPE cDNA insert (1473 bp) was ligated into an expression vector of pYES2 (Invitrogen, USA) to generate a construct containing the cDNA flanked with the yeast *GAL1* promoter and *CYC1* termination sequences of the plasmids, as described previously (Hiraiwa *et al.*, 1997b). Yeast *pep4* strain (YW7-6D; *MATa leu2 ura3-52 pep4-3*) of *Saccharomyces cerevisiae* (donated by Dr Y. Wada, Osaka University) was used as a host for transformation. Procedures for transformation and growth condition of the transformants were essentially the same as those described previously (Hiraiwa *et al.*, 1997b; Inoue *et al.*, 1997). Galactose-grown transformant cells were collected by centrifugation, sheared mechanically with glass beads (Sigma, St. Louis, MO, USA) and lysed in 20 mM Tris–HCl (pH 7.5) that contained 1 mM phenylmethanesulfonyl fluoride (PMSF), as described previously (Wada *et al.*, 1990). Whole cell lysates of the transformants were used for an assay of VPE activity.

Assay for vacuolar processing activity

VPE activity was assayed essentially as described previously (Hara-Nishimura *et al.*, 1991; Hiraiwa *et al.*, 1997b). A chemically synthesized decapeptide, Ser-Glu-Ser-Glu-Asn-Gly-Leu-Glu-Glu-Thr, was used as the substrate. The peptide sequence was derived from the sequence around the processing site of proglobulin, the proprotein precursor of 11S globulin, a major seed protein of pumpkin (Hayashi *et al.*, 1988). The reaction mixture contained 9 nmol of the decapeptide substrate and the lysate of the yeast transformant cells in 10 μl of 50 mM sodium acetate (pH 5.5), 50 mM dithiothreitol. The mixture was incubated for 0, 0.5 and 3.0 h at 37°C. After the addition of 25 μl of distilled water, the products of the reaction were subjected to analytical capillary electrophoresis (HP^{3D}, Hewlett-Packard GmbH, Germany) at 25°C and 30 kV in 10 mM sodium borate buffer (pH 9.2). Electrophoresis was monitored in terms of absorbance at 200 nm. The VPE cleaves only the peptide bond on the C-terminal side of the asparagine residue of the substrate decapeptide to generate an N-terminal pentapeptide P1 and a C-terminal pentapeptide P2.

In vivo assay of VPE activity was performed by monitoring maturation of CPY in the transformant cells of proteinase A-deficient (*pep4*) strain, as described previously (Hiraiwa *et al.*, 1997b). The transformant was grown in the galactose medium for 15 h. The cells collected were subjected to SDS-PAGE and subsequent immunoblot analysis with CPY-specific antibodies.

Preparation of specific antisera against γVPE

To prepare specific antibodies against γVPE, the DNA fragment of 1403 bp was inserted into an expression vector pET-32a (Novagen, Madison, WI, USA). The expressed histidine-tagged protein was subjected to SDS-PAGE followed by staining with Coomassie blue and the band corresponding to the γVPE fragment was cut out from the gel and injected subcutaneously into a rabbit with complete Freund's adjuvant. After 3 weeks, two booster injections with incomplete adjuvant were given at 7 day

intervals. One week after the booster injection, blood was drawn and the antiserum was prepared. Prepared antibodies against γVPE were used in an immunocytochemical analysis.

Immunocytochemical analysis

The 5th leaves of the 21-days-old plants that had been incubated for 48 h after the wound treatment were used. Procedures for ultrastructural studies and immunogold labeling were essentially the same as those described previously (Hara-Nishimura *et al.*, 1998a) except for the use of the anti-γVPE antibodies. The sections were treated with blocking solution (1% BSA in PBS) for 1 h at room temperature and then incubated overnight with anti-γVPE antibodies (1:500), in blocking solution at 4°C. After washing with PBS, sections were incubated for 30 min at room temperature with a solution of protein A-gold (10 nm; Amersham Japan) that had been diluted 1:30 in the blocking solution. For the control experiment, incubation of the section with the primary antibodies was omitted. The sections were washed with distilled water and were then stained with 4% uranyl acetate and lead citrate. After staining, all sections were examined with a transmission electron microscope (model 1200EX; JEOL, Tokyo, Japan) at 80 kV.

Acknowledgements

This work was supported by Grants-in-Aid for 'Research for the Future' Program (JSPS-RFTF96I60407), Scientific Research (nos. 08262220, 10440244 and 10163242) from the Ministry of Education, Science and Culture of Japan and a postdoctoral fellowship (T.K.) from the Japan Society for the Promotion of Science.

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Expression of a Vacuolar Protein (VP24) in Anthocyanin-Producing Cells of Sweet Potato in Suspension Culture[1]

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VP24, an abundant protein of 24 kD, was found to accumulate in the anthocyanin-containing vacuoles of cells of sweet potato (*Ipomoea batatas*) in suspension culture. Light-induced expression of VP24 was analyzed by immunoblotting in three different cell lines that produced anthocyanins at different rates. The expression of VP24 was closely correlated with the accumulation of anthocyanin in these cell lines. Immunocytochemical detection of VP24 with specific antibodies on thin sections showed that VP24 was localized in the intravacuolar pigmented globules (cyanoplasts) in the anthocyanin-containing vacuoles and not in the tonoplast. No VP24 immunogold labeling was detected in the vacuoles of the cell line that does not produce anthocyanin. We suggest that VP24 may be involved in the formation of the cyanoplast via an interaction with anthocyanin, and that it may play an important role in the trapping in vacuoles of large amounts of anthocyanins that have been transported into these vacuoles.

The central vacuole is the main site of accumulation of many water-soluble secondary metabolites such as anthocyanins and other phenolic compounds. It is generally accepted that the biosynthesis of anthocyanins and other flavonoid glycosides occurs in the cytosol and that these compounds are then transported to the central vacuole across the tonoplast (Hrazdina and Wagner, 1985; Hrazdina et al., 1987). The mechanisms for transport to the vacuoles have not been clearly established. However, it was reported recently that anthocyanin is conjugated with glutathione in a reaction that is catalyzed by glutathione *S*-transferase (encoded in maize by the *Bronze-2* gene) and that this conjugate is then transported to the vacuoles by the glutathione pump in the tonoplast (Marrs et al., 1995; Marrs, 1996).

Martinoia et al. (1993) showed that the uptake of glutathione *S*-conjugates of *N*-ethylmaleimide and of metolachlor into the vacuoles was mediated by a specific ATPase in the tonoplast, which was not linked to any known vacuolar proton pump. Furthermore, it has also been reported that naturally occurring metabolites such as cinnamic acid and other phenylpropanoid compounds are conjugated with glutathione in reactions catalyzed by glutathione *S*-transferase (Dean et al., 1995). An ATP-dependent glutathione pump in the vacuolar membrane may be part of the vacuolar membrane system that is involved in the transport of most phenolic compounds such as anthocyanins and other flavonoid glycosides.

Once the compounds are transported to the vacuoles, other mechanisms are necessary for intravacuolar retention (Guern et al., 1987). Several intravacuolar-trapping mechanisms, such as glycosylation, ionization, and induction of a conformational change, may be involved in the vacuolar accumulation of secondary metabolites (Werner and Matile, 1985; Matern et al., 1986). However, it remains unclear how large amounts of anthocyanin are retained in central vacuoles. In previous reports (Nozue and Yasuda, 1985; Nozue et al., 1987) we demonstrated that cultured cells of sweet potato (*Ipomoea batatas* Lam. cv Kintoki) produce large amounts of anthocyanin and form intensely pigmented, anthocyanin-containing globules within vacuoles during continuous illumination. These pigmented structures have been called cyanoplasts (Lippmaa, 1926; Molisch, 1928; Politis, 1959) and are first detected as small, red, spherical bodies approximately 3 μm in diameter at the early stage of pigment accumulation under light microscopy.

The number of these spherical bodies in the vacuole increases with the maximum accumulation of pigment, but it subsequently decreases to one or a few with an increase in the size of the pigmented bodies. Fully developed pigmented globules reach 10 to 15 μm in diameter. Electron microscopy has revealed that these intracellular structures have neither a membrane boundary nor an internal structure, and that they are found as strongly osmiophilic globules in vacuoles (Nozue et al., 1993). These observations indicate that the pigmented structures in anthocyanin-producing cells are insoluble globules in which anthocyanin is highly concentrated. The formation of such pigmented structures in anthocyanin-containing cells may

[1] This study was carried out under the auspices of the Cooperative Research Program (grant no. 94-103) of the National Institute for Basic Biology, Japan.

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Abbreviation: PAL, Phe ammonia-lyase.

represent the trapping of large amounts of anthocyanin in vacuoles.

Unpigmented spherical bodies were observed in the vacuoles of dark-grown seedlings of *Polygonum cuspidatum* (Kubo et al., 1995). The unpigmented spherical bodies accumulated anthocyanin and turned into cyanoplasts when dark-grown seedlings were irradiated. However, it is unknown how the cyanoplasts were formed in the vacuoles of anthocyanin-producing cells.

We recently described a 24-kD protein (VP24) that was identified as a major protein in vacuoles isolated from anthocyanin-producing cells of sweet potato in suspension culture (Nozue et al., 1995). Expression of VP24 was induced in cultured cells upon their exposure to light and was closely correlated with the accumulation of anthocyanin. Although the biological function of the vacuolar protein VP24 has not yet been determined, it seems possible that this protein may be involved in the intravacuolar accumulation of anthocyanins via the formation of cyanoplasts.

The present study was carried out to characterize in further detail the role of VP24 in the anthocyanin-containing vacuoles. We studied the expression and intravacuolar localization of this protein using three different lines of sweet potato cells in suspension culture, which produced anthocyanin at different rates.

MATERIALS AND METHODS

Cell Cultures

Three lines of sweet potato (*Ipomoea batatas* Lam. cv Kintoki) cells in culture, ALD, ALND, and N, were used in the present experiments. ALND and ALD were anthocyanin-producing cell lines and N was a nonproducing cell line. Anthocyanin content of this cell line was around the threshold of detectability. The ALND cell line produced large amounts of anthocyanin under continuous illumination but little anthocyanin in darkness. The ALD cell line produced large amounts of anthocyanin not only under continuous illumination but also in darkness. More anthocyanin was synthesized in ALD cells under illumination than in darkness. The N cell line produced little anthocyanin regardless of illumination conditions.

Both the ALND and N cell lines were obtained by clonal selection from the same callus cultures that had been initiated from tuberous root tissue of sweet potato (Nozue et al., 1987). The ALD cell line was also obtained by several rounds of clonal selection from the anthocyanin-producing cells that developed spontaneously in dark-grown cultures of ALND cells. All cell lines in callus culture were maintained in 25 mL of PRL-4C (Gamborg, 1966) agar medium that contained 3% Suc (w/v) and 0.3 mg L^{-1} 2,4-D in Petri dishes 9.0 cm in diameter in darkness at 25 to 26°C, with subculture at 2-week intervals. Seven-day-old callus of each cell line was used for the following experiments. Other procedures for the culture of these cells have been described previously (Nozue et al., 1995).

Induction of VP24 Expression and Anthocyanin Synthesis

Expression of VP24 and synthesis of anthocyanin were induced by exposure of cells to light, as described previously (Nozue et al., 1993). Callus (approximately 2 g fresh weight) of each cell line was transferred to a 100-mL Erlenmeyer flask that contained 20 mL of PRL-4C liquid medium without 2,4-D, unless otherwise stated. After preculture on a rotary shaker for 3 d in darkness, the cells were exposed to continuous illumination and harvested at various intervals after the onset of irradiation, and amounts of VP24 and anthocyanin were determined. To investigate the expression of VP24, N cells were cultured under identical conditions as ALND and ALD cells.

Extraction of VP24

Suspension-cultured cells were harvested at various intervals by filtration of the culture through filter paper (Toyo filter paper no. 1, Advantec, Tokyo, Japan) on a suction funnel and their fresh weight was determined. Cells (1 g fresh weight) were homogenized with a mortar and pestle in 1 mL of extraction buffer (25 mM Tris-Mes, pH 6.8, containing 0.5 M NaCl, 0.3 M Suc, and 3 mM $MgCl_2$) with 0.1 g of Polyclar AT and 1 g of quartz sand at 0°C. The homogenate was centrifuged at 12,000*g* for 10 min at 4°C, and the supernatant was stored at −20°C prior to analysis.

Protein was precipitated with sodium deoxycholate and TCA (Bensadoun and Weinstein, 1976) and dissolved in 1 N NaOH, and concentration was determined by the method of Lowry et al. (1951). BSA was used as a standard.

Electrophoresis and Immunoblotting

One-dimensional SDS-PAGE was performed as described by Laemmli (1970). Samples were heated at 95°C for 3 min in the presence of 1% SDS and 1% 2-mercaptoethanol, and were then subjected to SDS-PAGE in a 14% (w/v) polyacrylamide gel for separation and in a 4.5% (w/v) polyacrylamide gel for concentration. Two-dimensional gel electrophoresis was carried out as described by O'Farrell (1975) with IEF (2% Ampholine 3.5–10, Pharmacia) in the first dimension, and SDS-PAGE (14% gel) in the second dimension.

For immunoblotting, the proteins were blotted electrophoretically onto a PVDF membrane and were detected with VP24-specific polyclonal antibodies as the primary antibody (1:1000 dilution) and goat antibodies conjugated with alkaline phosphatase against rabbit IgG (Cappel) as the second antibody. VP24 was quantified densitometrically after immunoblotting. Polyclonal antibodies were raised in a rabbit against a purified VP24 preparation that had been extracted from vacuoles isolated from suspension-cultured ALND cells and purified by preparative SDS-PAGE.

Quantitation of Anthocyanin

Samples (0.1 g fresh weight) were extracted for 24 h with 5 mL of methanol (0.5% HCl, v/v) at 4°C in darkness. After

Table I. *Anthocyanin content of callus-cultured sweet potato cell cultures*

Callus was cultured on PRL-4C agar medium supplemented with 0.3 mg L^{-1} 2,4-D for 12 d under continuous illumination or in darkness.

Culture Conditions	Anthocyanin Content		
	ALD	ALND	N
	μmol g^{-1} fresh wt		
Light	4.7	7.9	ND[a]
Dark	1.2	ND	ND

[a] ND, Below 10 nmol g^{-1} fresh weight.

removal of insoluble materials by centrifugation at 1,600*g* for 10 min, the A_{530} of the clear supernatant was measured and the anthocyanin concentration was determined using $\epsilon = 33{,}000\text{-l-mol}^{-1}\text{-cm}^{-1}$ (Moskowitz and Hrazdina, 1981).

Preparation of Vacuoles

Protoplasts and vacuoles were isolated from 8-d-old suspension-cultured ALD cells as described previously (Nozue et al., 1993, 1995).

Immunocytochemical Analysis

Fourteen-day-old suspension-cultured ALD cells that had been transferred to liquid medium without 2,4-D were harvested by centrifugation and vacuum infiltrated for 1 h with a fixation mixture that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 60 mM Suc in 50 mM cacodylate buffer, pH 7.4. Cells were washed with 50 mM cacodylate buffer, pH 7.4, dehydrated in a graded dimethylformamide series at −20°C, and embedded in London Resin White acrylic resin (London Resin Co. Ltd., Basingstoke, Hampshire, UK). Blocks were polymerized under a UV lamp at −20°C for 24 h. Ultrathin sections were prepared on a Reichert ultramicrotome (Leica, Heidelberg, Germany) and mounted on uncoated Ni^{2+} grids. Immunocytochemical labeling with protein A gold particles was performed as described previously (Nishimura et al., 1993). Antibodies against VP24 (dilution 1:1000) were used for the analysis. Thin sections were examined with a transmission electron microscope (1200 EX, Jeol) operated at 80 kV.

Assay for PAL Activity

Cultured cells (0.5 g fresh weight) were homogenized with a mortar and pestle in 2 mL of 0.1 M Tris-HCl buffer, pH 8.0, that contained 20 mg of sodium isoascorbate, 50 mg of Polyclar AT, and quartz sand at 0°C. The homogenate was filtered through Miracloth (Calbiochem) and the filtrate was centrifuged at 12,000*g* for 10 min. The supernatant was partitioned on a Sephadex G-25 column (Pharmacia), and the protein in the eluate was used for assay of PAL activity as described by Tanaka et al. (1974). A unit of activity was defined as the amount of enzyme catalyzing the formation of one nanomole of cinnamic acid per minute.

RESULTS

Expression of VP24 and Anthocyanin in Three Cell Lines

Marked increases in levels of both VP24 and anthocyanin were found in vacuoles isolated from ALND cells that had been cultured under continuous illumination (Nozue et al., 1995). However, neither VP24 nor anthocyanin was detected in dark-grown-cultured cells. To compare the abilities of three cell lines to synthesize VP24, we examined the expression of VP24 and the formation of anthocyanin in the ALD, ALND, and N cells. Callus cultures of each cell line were harvested after 12 d of culture under continuous illumination or in darkness. The anthocyanin content was then determined and VP24 was analyzed by SDS-PAGE with subsequent immunoblotting.

Table I shows that large amounts of anthocyanin were produced in both the irradiated and dark-cultured ALD cells, and that anthocyanin was also produced in the irradiated ALND cells. However, little anthocyanin was produced in the dark-cultured ALND cells. The N cells failed to produce any anthocyanin in light or in darkness. Figure 1 shows that large amounts of VP24 were expressed in both ALD and ALND cells, and that a small amount of VP24 was detected in N cells grown in the light. However, no VP24 was detected in ALND and N cells cultured in darkness. The dark-cultured ALD cells expressed VP24 and synthesized anthocyanin. Larger amounts of both VP24 and anthocyanin were produced in the irradiated ALD cells than in the dark-cultured ALD cells.

Light-Induced Expression of VP24 and Anthocyanin

Changes in levels of VP24 in the three different cell lines after light irradiation were examined by immunoblot analysis. Suspension-cultured cells of each line were initiated by transfer of callus to 2,4-D-free liquid medium and then cells were cultured in darkness prior to irradiation. Figure 2 shows the changes in the relative amount of VP24 produced by the suspension-cultured cells of each line upon exposure to light. ALD cells expressed a certain level of VP24 continuously during culture in darkness. A slight

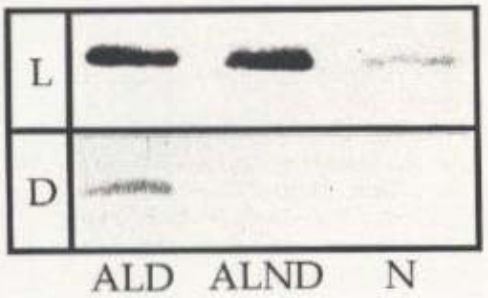

Figure 1. The expression of VP24 in the three cell lines ALD, ALND, and N of sweet potato callus. VP24 was analyzed by SDS-PAGE (5 μg of protein per lane) with subsequent immunoblotting with antibodies against VP24. Each callus was cultured on PRL-4C agar medium that contained 3% Suc and 0.3 mg L^{-1} 2,4-D for 12 d under continuous light (L) or in the darkness (D).

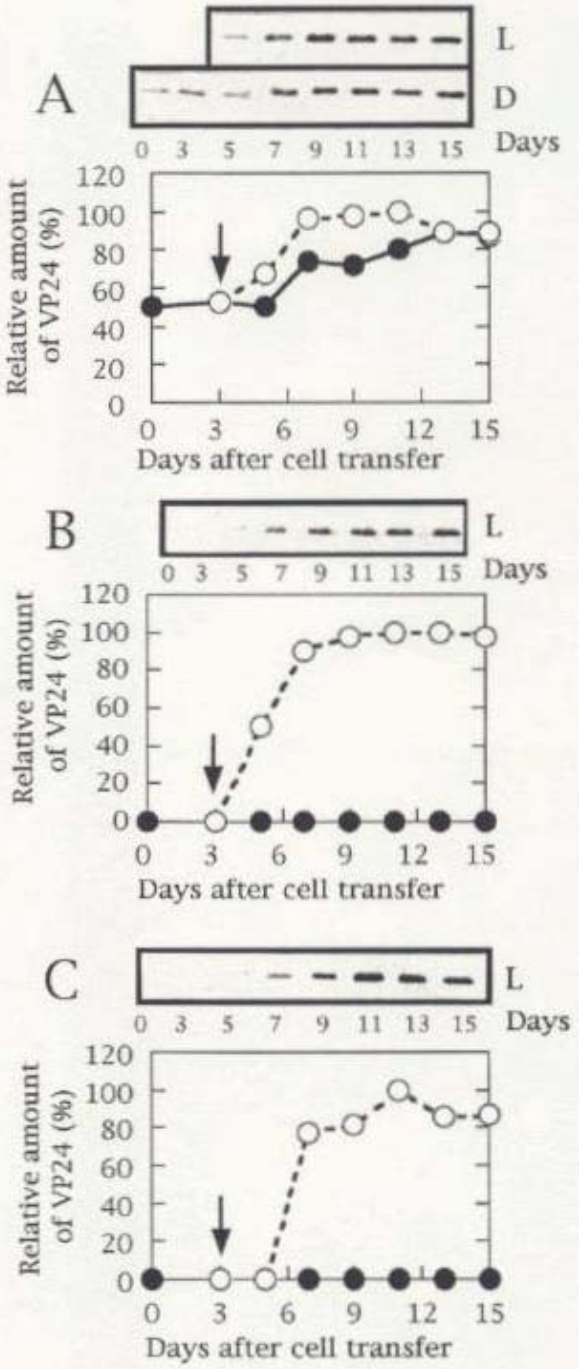

Figure 2. Changes in levels of VP24 in suspension-cultured cells of three cell lines. Suspensions of cells were initiated from the callus of each cell line, ALD (A), ALND (B), and N (C), by transfer to 2,4-D-free liquid medium and then cultured in darkness (●). Cells were irradiated 3 d after transfer (arrow) and then cultured under continuous illumination (○). The relative amounts of VP24 were measured by densitometric analysis of the corresponding bands on the immunoblots shown in the upper panels of each figure, and are expressed as a percentage of the value of the 11-d-old cells under continuous illumination. Cell extracts (5 μg of protein per lane) were fractionated by SDS-PAGE, with subsequent immunoblotting analysis with antibodies against VP24. The numbers below the upper panel in each figure indicate days after transfer. L and D are as in Figure 1.

increase in the level of VP24 was observed at the late stage of cultures in darkness, but the level of VP24 was enhanced by light irradiation (Fig. 2A). In contrast, little VP24 was detectable in dark-cultured ALND and N cells, but the level of VP24 in both cell lines increased markedly upon irradiation (Fig. 2, B and C).

Figure 3 shows the changes in anthocyanin content under the same conditions as those in the experiment for which results are shown in Figure 2. Light-stimulated or -induced synthesis of anthocyanin was observed in ALD and ALND cells (Fig. 3, A and B). The increase in anthocyanin content in both cell lines under continuous illumination occurred more slowly than that of VP24, and more than 10 d of irradiation were required to reach the maximum pigment level. Anthocyanin synthesis occurred continuously in the dark-cultured ALD cells, but was stimulated by irradiation. Little anthocyanin accumulated in

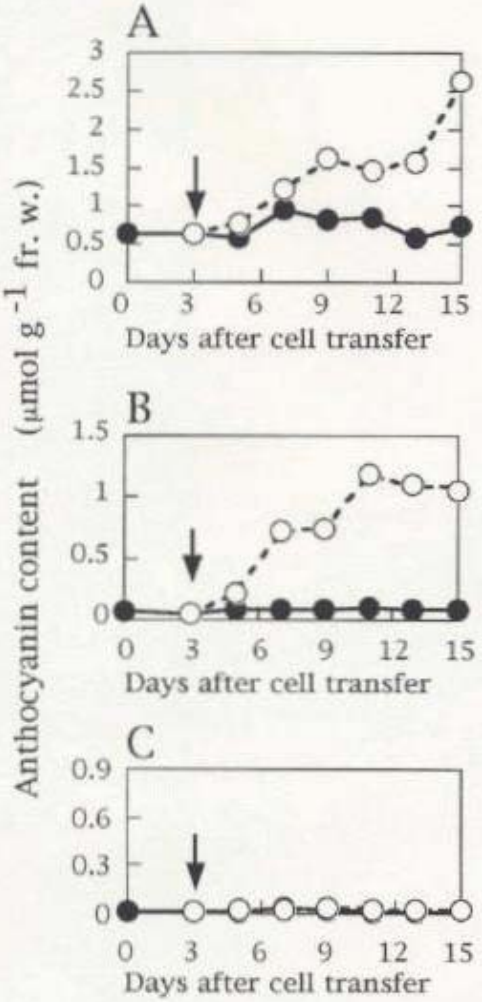

Figure 3. Changes in the anthocyanin content of suspension-cultured cells derived from the ALD (A), ALND (B), and N (C) callus. Anthocyanin content was measured using the same suspension-cultured cells as those examined in the experiments for which results are given in Figure 2. Arrows indicate the start of light irradiation 3 d after transfer of callus cells to liquid medium. For symbols, see legend to Figure 2.

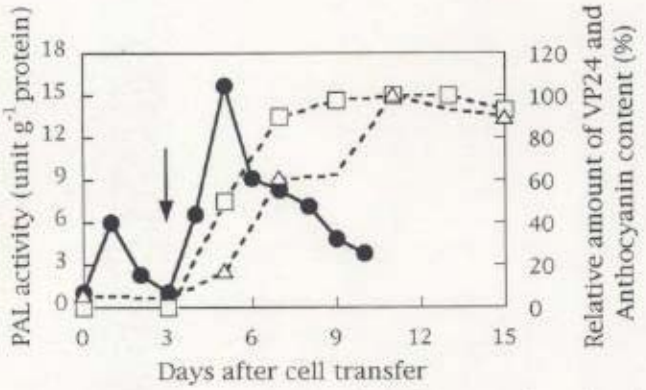

Figure 4. Changes in the activity of PAL in suspension-cultured ALND cells. Suspension cultures were initiated from callus, and cells were cultured under the same experimental conditions as described in the legends to Figures 2 and 3. The dark-cultured cells were irradiated 3 d after cell transfer (arrow) and then cultured under continuous illumination. PAL activity (●) and the relative amounts of VP24 (□) and anthocyanin (△) are shown. The values of VP24 and anthocyanin are expressed as a percentage of the value of the 11-d-old cells.

irradiated or dark-cultured N cells (Fig. 3C). These results indicate that stronger expression of VP24 was accompanied by greater accumulation of anthocyanin in the cultured cells of all three cell lines, with the exception of the irradiated N cells.

PAL is a key enzyme in the biosynthesis of phenylpropanoids and phenylpropanoid-derived compounds. The changes in PAL activity in ALND cells were examined under the same experimental conditions as described above for a comparison with the changes in levels of VP24. A transient increase in PAL activity after irradiation of cells was observed and it was followed by the increase in activity that is known to be induced by the cell transfer to fresh medium (Hahlbrock and Schröder, 1975). PAL activity reached a maximum 2 d after the start of light irradiation and then decreased rapidly (Fig. 4). The accumulation of anthocyanin began after the increase in PAL activity and paralleled that of VP24. Both VP24 and anthocyanin reached maximum values after the decrease in the activity of PAL.

Effect of 2,4-D on the Light-Induced Expression of VP24

Elevated concentrations of auxins usually inhibit the synthesis of anthocyanin and other phenolic compounds in plant cells in culture (Constabel et al., 1971; Ozeki et al., 1987). As reported previously (Nozue and Yasuda, 1985), 2,4-D markedly inhibited the synthesis of anthocyanin and the development of cyanoplasts in cultured cells of sweet potato. The level of VP24 in the isolated vacuoles of ALND cells was reciprocal with increases in the concentration of 2,4-D (Nozue et al., 1995). To study the correlation between the expression of VP24 and the formation of anthocyanin, we examined the effects of 2,4-D in three different cell lines.

Callus in each cell line was transferred to liquid medium that contained 2,4-D at various concentrations, and cells were cultured under continuous illumination after 3 d of culture in darkness. Cells were harvested 14 d after the start of irradiation. Figure 5 shows the effects of 2,4-D on the expression of VP24 and the amount of anthocyanin in the illuminated cells. The level of VP24 in all cell lines was reduced by the presence of elevated concentrations of 2,4-D, particularly in ALND cells. More than 0.3 mg L^{-1} 2,4-D markedly inhibited the light-induced expression of VP24, and more than 0.1 mg L^{-1} inhibited the synthesis of anthocyanin. However, both the expression of VP24 and the synthesis of anthocyanin in ALD cells were inhibited

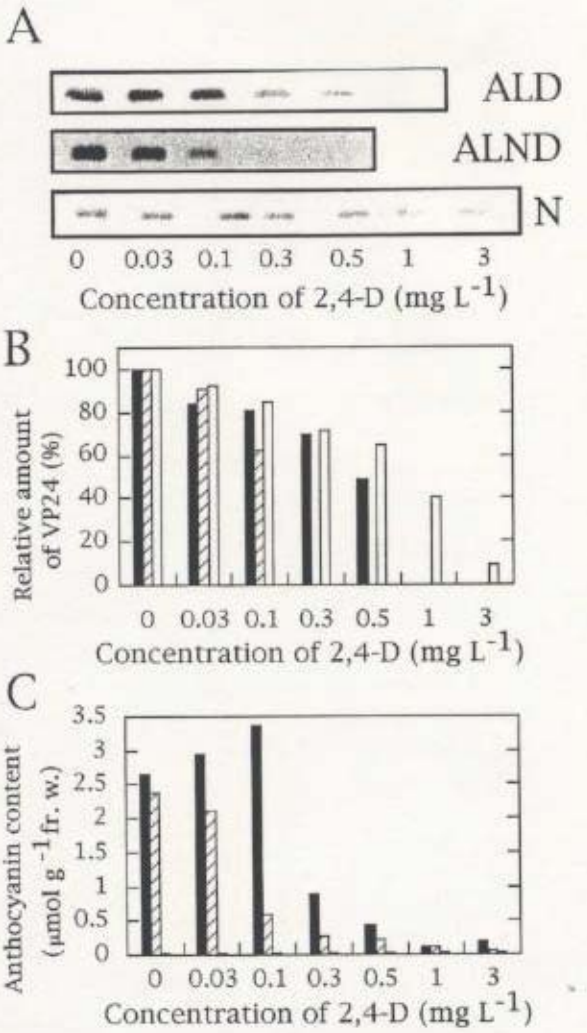

Figure 5. Effects of 2,4-D on the accumulation of VP24 and anthocyanin in suspension-cultured cells of the three lines. Calli of each cell line were transferred to liquid medium supplemented with 2,4-D at various concentrations, and then cells were cultured under continuous illumination after keeping in dark for 3 d. Cells were harvested 14 d after the start of irradiation. Cell extracts (5 μg of protein per lane) were separated by SDS-PAGE, followed by immunoblotting and analysis with antibodies against VP24 (A). Relative amounts of VP24 (B) were determined by densitometric analysis of the corresponding bands on the immunoblot shown in A and expressed as a percentage of the value of the cells cultured without 2,4-D medium under continuous illumination, and anthocyanin (C) was measured as described in the text. ■, ALD; ▨, ALND; □, N.

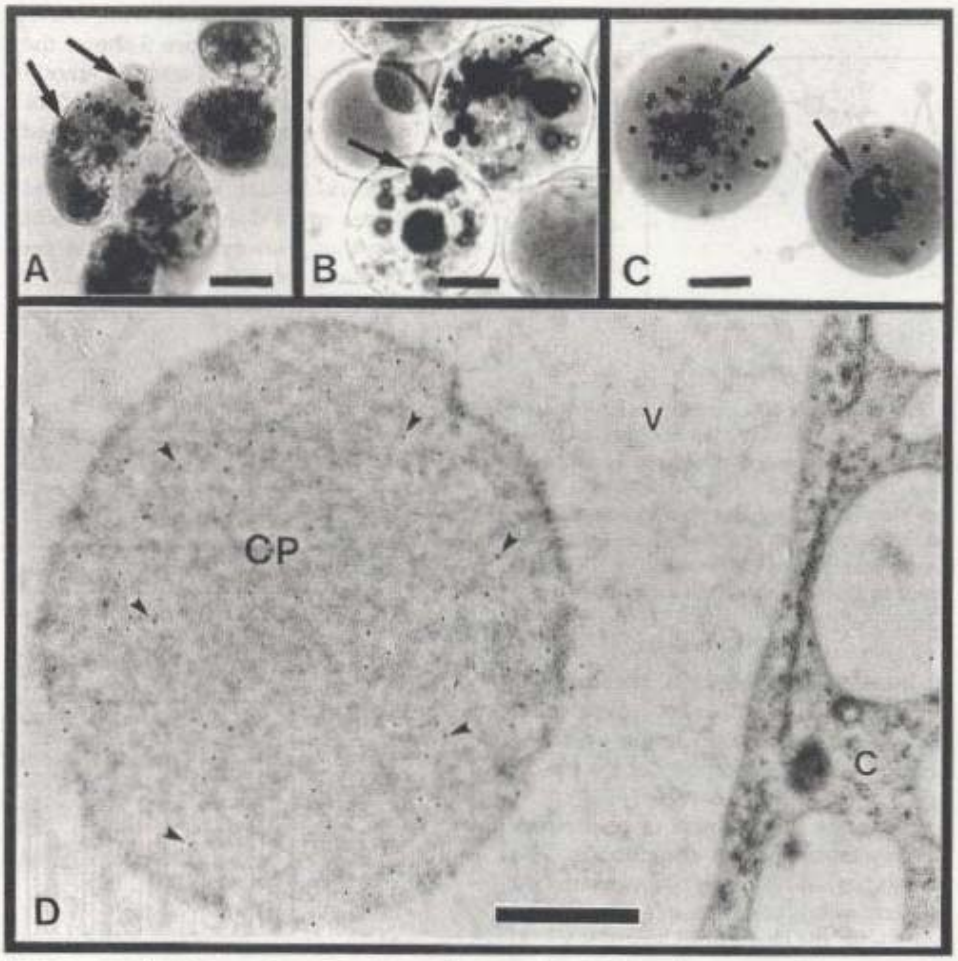

Figure 6. The intravacuolar pigmented structures (cyanoplasts) in cultured ALD cells (A), protoplasts (B), and vacuoles (C), as observed by light microscopy. Protoplasts and vacuoles were isolated from ALD cells that had been cultured for 8 d under continuous illumination. Arrows indicate cyanoplasts and bars are 20 μm. D, Immunocytochemical detection of VP24 in ALD cells that had been cultured for 14 d under continuous illumination. Arrowheads indicate immunogold particles. Bar is 1 μm. CP, Cyanoplast; V, vacuole; C, cytosol.

less strongly by 2,4-D. The expression of VP24 in N cells was not completely inhibited by 2,4-D.

Localization of VP24

The knowledge of a protein's subcellular localization is essential for functional studies. VP24 was identified as a major protein in vacuoles isolated from anthocyanin-containing cells of sweet potato (Nozue et al., 1995). However, we have no direct evidence to indicate whether VP24 is a tonoplast-integrated protein or an intravacuolar-soluble protein. To study the subcellular localization of VP24 in anthocyanin-containing and anthocyanin-free cells, we subjected suspension-cultured ALD and N cells to immunocytochemical analysis of VP24. Both ALD and N cells expressed VP24 in the light, but production of anthocyanin only occurred in ALD cells. Figure 6, A through C, shows the cyanoplasts in the cultured ALD cells and in protoplasts and vacuoles isolated from ALD cells that had been cultured for 8 d under continuous illumination.

Large numbers of cyanoplasts were found in the vacuoles of the sweet potato cells in suspension culture, as reported previously (Nozue et al., 1993). Immunocytochemical staining and electron microscopy revealed that VP24 was localized in the cyanoplasts and vacuoles of ALD cells and not in the tonoplast (Fig. 6D). This suggests an association of VP24 with anthocyanin pigments. Neither the accumulation of anthocyanin nor the occurrence of cyanoplasts was observed in N cells, but VP24 was found as a vacuolar protein (Fig. 1). We tried to detect VP24 in the vacuoles of N cells. However, we were unable to identify any immunogold-labeled structures in these cells (data not shown).

DISCUSSION

The synthesis of anthocyanin in cultured plant cells is generally stimulated or induced by illumination. In the present study we used three lines of sweet potato cells in

cultures with different sensitivities to light with respect to the formation of anthocyanin. These cell lines were established by several rounds of clonal selection from the same callus that had been initiated from sweet potato root. It is unclear why the ability to produce anthocyanin differed among the three cell lines. However, the activities of enzymes involved in general phenylpropanoid metabolism, PAL, 4-coumarate:CoA ligase, and cinnamate 4-hydroxylase, in N cells were undetectable or very low under continuous illumination (M. Nozue and S. Kuwazawa, unpublished data; M. Nozue and S. Katajir, unpublished data).

In contrast, the activities of these enzymes in ALD cells cultured in darkness were high, and large amounts of anthocyanin were produced and accumulated. These results indicate that the expression of VP24 in ALD cells is correlated with the accumulation of anthocyanin, as is the case in ALND cells (Nozue et al., 1995). Furthermore, the inhibitory effects of 2,4-D on the accumulation of VP24 also showed the same tendencies as the inhibitory effects of 2,4-D on the intravacuolar accumulation of anthocyanin in these cell lines.

The activities of the enzymes that are related to general phenylpropanoid metabolism and the biosynthesis of flavonoids usually show a transient increase soon after induction by various triggers (Lawton et al., 1983; Chappell and Hahlbrock, 1984; Ozeki et al., 1990; Peter et al., 1991). A transient increase in the activity of PAL was also observed before the level of anthocyanin reached a maximum in cultured cells of sweet potato. However, the level of VP24 continued to increase, as did the accumulation of anthocyanin. It is generally believed that the biosynthesis of anthocyanin is a cytosolic event and that anthocyanin is transported to the vacuole (Hrazdina et al., 1978; Jonsson et al., 1983; Marrs et al., 1995). Our observations indicate that VP24 may be involved in the accumulation of anthocyanin but not in its synthesis.

Electron microscopy after immunostaining of VP24 revealed that it is an intravacuolar and not a tonoplast-integrated or a tonoplast-associated protein, and is localized in the cyanoplasts of anthocyanin-containing vacuoles. Cyanoplasts were not formed in the cell line that did not produce anthocyanin in the light (N), but VP24 was detectable by SDS-PAGE with subsequent immunoblotting. VP24 may have been localized in the vacuole as a soluble protein, since neither intravacuolar osmiophilic globules (cyanoplasts) nor immunogold-labeled VP24 could be detected in the N cells. Cyanoplasts were detectable only in cells that accumulated large amounts of anthocyanin.

The factors affecting the occurrence of cyanoplasts in cultured sweet potato cells coincided with those that influenced the expression of VP24 and the accumulation of anthocyanin. Both the occurrence of cyanoplasts (Nozue and Yasuda, 1985) and the expression of VP24 in the cultured cells were strongly inhibited by 2,4-D in the medium. These results indicate that VP24 may be involved in the formation of cyanoplasts and that this vacuolar protein coexists with anthocyanins in the same vacuoles. As described previously (Nozue et al., 1995), VP24 was recovered from only the reddish or purplish pellet after ultracentrifugation of sonicated samples of isolated vacuoles. It seems that VP24 combines easily with anthocyanins in vitro. Two-dimensional gel electrophoresis indicated that VP24 was an acidic protein with a pI below 5.5, and VP24 was barely soluble in the buffered solutions below pH 5.5 (data not shown). Therefore, VP24 may not be in solution at the vacuolar pH. These observations suggest that VP24 may play a role in the intravacuolar trapping of anthocyanin pigments via the formation of cyanoplasts through hydrophobic interaction with anthocyanin in vivo.

VP24 may have other biological roles in addition to its role in the formation of cyanoplasts, because this protein also accumulated in the vacuoles of nonproducing N cells. The present study suggests that the mechanisms that regulate the expression of VP24 and the formation of anthocyanin in sweet potato cells may be similar, but the expression of VP24 may be independent of the synthesis of anthocyanin. Although the control of the expression of VP24 has not yet been characterized at the molecular level, our preliminary experiments with inhibitors of RNA and protein synthesis indicate that the light-stimulated or light-induced expression of VP24 may be regulated at the transcriptional level. When the suspension-cultured ALND cells were treated with cycloheximide or cordycepin before and after the start of irradiation of cells, the expression of VP24 was inhibited by treatment with either inhibitor before the start of irradiation, but little inhibition was observed in cells that had been treated more than 3 d after the start of irradiation.

The central vacuole in the mature plant cell is generally the largest subcellular compartment, and it is the site of storage of a variety of compounds including proteins. VP24 is distinct from sporamin, a major vacuolar storage protein of 25 kD in the tuberous roots of sweet potato (Maeshima et al., 1985). However, no sporamin was detectable in an extract of cultured sweet potato cells by immunoblotting (Nozue et al., 1995). It was reported that the pathogenesis-related proteinase P69 (Vera and Conejero, 1988; Vera et al., 1989) and a salt-stress protein, osmotin (Singh et al., 1987), were localized as electron-dense inclusion bodies within the vacuoles of tomato leaf and cultured tobacco cells, respectively. Considerable amounts of these proteins accumulated in the vacuoles.

Although the function of VP24 is unknown, the properties of the protein are clearly different from those of P69 and osmotin. Recently, a carboxypeptidase associated with electron-dense inclusions in the vacuoles (Mehta et al., 1996) was isolated from tomato fruit and characterized (Mehta and Mattoo, 1996). P69 and the carboxypeptidase are cationic proteins, and immunocytochemical staining and electron microscopy indicated that both proteins are presumed to be amorphous aggregates, unlike the spherical cyanoplasts. The aminoterminal sequence of the VP24 polypeptide was determined (data not shown), but the sequence did not correspond to that of any known protein. VP24 may be a new, unique vacuolar protein. Further biochemical and molecular characterization of VP24 will be necessary to understand the function of this protein in sweet potato and other plants.

ACKNOWLEDGMENTS

The authors thank Drs. Takashi Hashimoto and Masako Miyazawa for their helpful advice and assistance in the preparation of antibodies.

Received June 30, 1997; accepted July 24, 1997.

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貯蔵たん白質の細胞内輸送と成熟化の機構の解析

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Vacuolar Sorting Machinery and Processing Mechanism for Storage Proteins

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ABSTRACT

Novel vesicles that accumulate large amounts of proprotein precursors of storage proteins were purified from developing pumpkin seeds and were designated precursor-accumulating (PAC) vesicles. The PAC vesicles mediate the transport of the precursor of a major storage protein (pro2S albumin) to protein-storage vacuoles in the developing seeds. We characterized two homologous proteins from PAC vesicles, a 72-kDa protein (PV72) and an 82-kDa protein (PV82). PV72 and PV82 showed an ability to bind to peptides derived from both an internal propeptide and a C-terminal peptide of pro2S albumin. PV72 was predicted to be a type I integral membrane protein with epidermal growth factor (EGF)-like motifs. These results suggest that PV72 and PV82 are potential sorting receptors for 2S albumin to protein-storage vacuoles. In the next step, we characterized a 100-kDa component (PV100) of the vesicles. Isolated cDNA for PV100 encoded a 97, 310-Da protein that was composed of a hydrophobic signal peptide and the following three domains: an 11-kDa Cys-rich domain with four CxxxC motifs (C, Cys), a 34-kDa Arg/Glu-rich domain composed of six homologous repeats, and a 50-kDa vicilin-like domain. Molecular characterization showed that two Cys-rich peptides, three Arg/Glu-rich peptides and the vicilin-like protein were produced by cleaving Asn-Gln bonds of PV100 by vacuolar processing enzyme (VPE) and that all these proteins had a pyroglutamate at their NH_2 terminus. Our findings suggested that VPE was responsible for cleaving Asn-Gln bonds of a single precursor, PV100, to produce multiple seed proteins in the vacuoles of seed cells. *Soy Protein Research, Japan* **1**, 6-12, 1998.

Key words : PAC vesicles, vacuolar sorting receptor, developing seeds, storage protein, 2S albumin, vacuolar processing enzyme

重要な食糧源のひとつである種子の貯蔵たん白質は，登熟期の種子細胞の粗面小胞体で合成され，液胞へ輸送されて蓄積される[1-3]．種子の細胞に見出される液胞は、葉や茎などの栄養器官の細胞に存在する分解型の

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液胞（LV; lytic vacuoles）とは区別され、たん白質蓄積型（PSV; protein-storage vacuoles）と呼ばれている．粗面小胞体で合成されたたん白質はどのようにしてたん白質蓄積型液胞へ運ばれるのであろうか？　液胞たん白質の選別輸送の機構を解析することは，種子の改良を目指す上で重要な課題となる．必須アミノ酸含量の高い改変貯蔵たん白質遺伝子を導入した形質転換体を作製する場合，細胞内で発現されたたん白質が正しく液胞へ輸送され，蓄積されなければならない．

著者らは，カボチャ貯蔵たん白質の細胞内輸送に関与する小胞を見出し，PAC（precursor-accumulating）小胞と命名した[4]．PAC小胞は直径300～500 nmの小胞で，動物のリソソームたん白質や酵母の液胞たん白質の輸送に関わっていることが知られているcoated vesicles（直径50～70 nm）とは明らかに異なる[4]．種子の貯蔵たん白質の細胞内輸送には，独特の経路が使われていることが示されつつある[4,5]．PAC小胞は，貯蔵たん白質の前駆体を多量に含んでおり，これらの前駆体をたん白質蓄積型液胞へ効率良く輸送する[6,7]．このことは，PAC小胞には貯蔵たん白質を液胞へ選別輸送するための装置が存在することを示唆している．この装置の同定を目的として，著者らは，登熟期の種子よりPAC小胞の単離に成功し，その構成成分の解析を行った．

上記の研究の結果明らかになってきた次の2点：（1）種子貯蔵たん白質の細胞内選別輸送に関わるPAC小胞とその膜に局在する種子たん白質前駆体の選別レセプター，（2）一つの種子たん白質前駆体から複数の成熟型たん白質を生成する新規の成熟化機構について述べる．

方　　法

PAC小胞の単離と72/82 kDaたん白質の解析[8]

開花後22～28日目のカボチャの子葉（約10～20 g）を抽出液（20 mM sodium pyrophosphate, pH 7.5, 1 mM EDTA, 0.3 M mannitol）で破砕後遠心（3,000 *g*, 15分）する．この上清を再び遠心（8,000 *g*, 20分）し，沈殿画分を1 mLの溶液（10 mM Hepes-KOH, pH 7.2, 1 mM EDTA, 0.3 M mannitol）に混ぜ，28%パーコールの上に重層して，パーコールの自己密度勾配遠心（40,000 *g*, 30分）により，PAC小胞を精製した[4,9]．単離小胞は，電子顕微鏡観察および指標酵素活性測定により他のオルガネラや細胞基質成分の混在がないことを確かめた[4]．

単離PAC小胞をSDS電気泳動後，72 kDaと82 kDaのバンドのN末端アミノ酸配列を決定し，それぞれをPV72とPV82と命名した[8]．PV72のアミノ酸配列を基に，登熟カボチャ子葉のcDNAライブラリーよりPV72をコードしているcDNAを単離し，構造解析を行った．

2Sアルブミン前駆体由来のペプチドに対するPV72/82の結合活性の測定[8]

カボチャの主要貯蔵たん白質の一つ2Sアルブミンに注目し，そのプロペプチド領域とC末端領域のペプチドを合成し，これをリガンドとするアフィニティカラムを作製した（2 mL of a bed volume of Aminolink gels; Pierce, Rockford, IL, U.S.A.）．上記のペプチドの各アミノ酸残基に変異を入れたものを合成して，同様のカラムを作製した．分解型液胞たん白質の選別輸送シグナルとして知られているオオムギのアリューレインのNPIR配列を含むプロペプチドも合成し，同様の実験に使用した．

一方，登熟カボチャ子葉よりミクロソーム画分を遠心分画により調製し[10]，超音波処理後，膜画分を遠心（100,000 *g*, 60分）で沈殿させた．この膜画分より，PV72/82を1%（w/v）3-[(3-cholamidopropyl dimethylammonio]-1-propanesulfonate（CHAPS）と10%（v/v）glycerolを含む溶液で可溶化する．この抽出液をPV72/82の粗抽出液として，上記のアフィニティカラムにかけ，カラムを洗浄後にリガンドに用いたペプチドで溶出した．

PAC小胞の100 kDaたん白質の解析PV100[11]

PAC小胞の主要構成成分の一つ100 kDaのバンド（PV100）のN末端アミノ酸配列を決定し，この配列を基にPV100のcDNAを単離した．cDNAの構造解析を行った．

結果と考察

PAC小胞の膜たん白質PV72の構造

Fig. 1は単離精製したPAC小胞のSDS電気泳動パターンの高分子量領域である．PAC小胞は，種子貯蔵たん白質11Sグロブリンの前駆体プログロブリン（pG）のほか，PV72/PV82やPV100（後述）を含んでいることが分かる．PV72とPV82のN末端アミノ酸配列はそれぞれ，RFVVEKNSLKVTYPDSIKGVYEXAIGNFGVPEYGGTMTとXFVVEKNSLRVTSPERIRGTと決定され，両者は互いに類似していた．Fig. 2は，単離されたPV72のcDNAから推定された一次構造とシロイヌナズナより単離されたホモログ（AtELP）[12]との構造比較である．PV72とAtELPはアミノ酸レベルで74%の相同性を示した．PV72前駆体はN末端に疎水性のシグナルペプチドを持っている．PV72の構造の特徴は，(1) 成熟型のPV72分子のC末端領域に膜貫通ドメイン

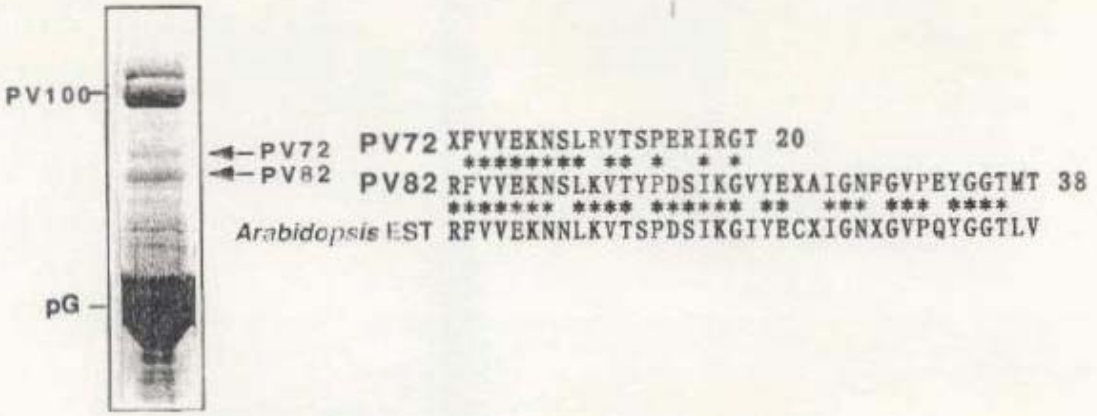

Fig. 1. Protein components of the PAC vesicles. The PAC vesicles (100 μg) were subjected to SDS-PAGE (8% acrylamide) and then to staining with CBB to detect PV72 and PV82. Comparison of N-terminal amino acid sequences of PV82 (20 residues) and PV72 (38 residues) with the sequence of an *Arabidopsis* EST clone (EMBL accession number Z38123). PV100 indicates an 100-kDa major protein of PAC vesicles. pG represents proglobulin, a proprotein precursor of 11S globulin.

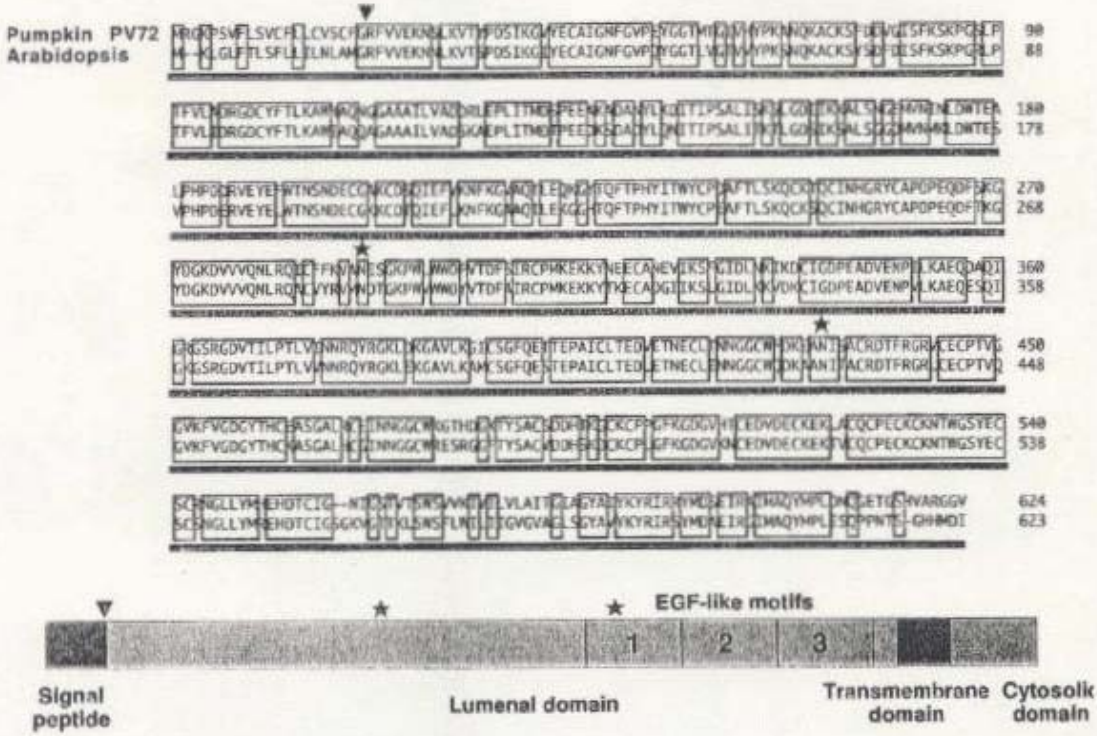

Fig. 2. Alignment of pumpkin PV72 with *Arabidopsis* homolog (AtELP) and structural characterization of PV72. A possible site of signal peptide cleavage is indicated by an arrowhead. Three EGF-like motifs are numbered (starting at positions 413, 466 and 514). Two possible glycosylation sites are indicated by asterisks.

を有するタイプI型の膜たん白質であること，(2) この膜貫通ドメインを境にして短い細胞基質ドメインと大きな小胞内ドメインに分かれること，(3) 後者のドメインにはシステイン含量の高い epidermal growth factor (EGF) 様モチーフが3回繰り返していることである．

上記の構造の特徴はエンドウの BP-80[13] にもみられる．BP-80 は，分解型液胞に局在するシステインプロテイナーゼであるアリューレインの液胞輸送シグナルである NPIR 配列と結合することが既に報告されている[14]．Fig. 3 は，NPIR 配列を含むアリューレインのプロペプチド (PAP) をリガンドとするアフィニティカラムに PV72/82 が特異的に結合し，溶出されてくるパターンを示している．これにより，PV72/82 も液胞たん白質の選別輸送のレセプターである可能性が示唆された．

しかしながら，PAC 小胞は，分解型液胞たん白質のための輸送小胞ではない．PAC 小胞は，内部に種子貯蔵たん白質の前駆体を多量に蓄積しており，これらを液胞へ輸送する[3,4,6,7]．次のステップとして，PV72/82 がこれらの貯蔵たん白質の選別輸送のためのレセプターとして機能するか否かを調べた．

PV72/82 は 2S アルブミン前駆体由来のプロペプチドに結合する

2S アルブミンは，前駆体プロ 2S アルブミンとして合成の場である粗面小胞体からたん白質蓄積型液胞へ輸送される．プロ 2S アルブミンは N 末端領域，中間領域と C 末端領域にプロペプチドを持ち，これらのペプチドは液胞内で除去されて成熟型の 2S アルブミンとなることが知られている[15]．カボチャ 2S アルブミン[3] の N 末端プロペプチド (2S−N)，中間プロペプチド (2S−I) および C 末端ペプチド (2S−C) の3種類のペプチドの各々をリガンドとするアフィニティカラムに PV72/82 の粗抽出液をかけた．その結果，2S−I と 2S−C に特異的に結合することが分かった (Fig. 4)．次に，2S−I と 2S−C の一部の残基をグリシンに変えた変異ペプチドをリガンドとしてアフィニティクロマトを行った結果，2S−I の RRE 配列と 2S−C の NSPL 配列が PV72/82 との結合に必須であることが分かった．以上の結果から，PV72/82 が，たん白質蓄積型液胞への種子たん白質の選別輸送に関わっていることが明らかになった．

PAC 小胞の構成成分 PV100 の構造解析

PAC 小胞によって様々な種子たん白質が前駆体の形で液胞へ輸送される[3,4]．これらのたん白質の多くは液胞内に局在している液胞プロセシング酵素の働きで成熟型に変換する[16-22]．PV100 もやはり液胞プロセシング酵素によって成熟型に変換する種子たん白質の前駆体であったが，興味深いことに，PV100 はプロセスされることによって，複数の種子たん白質を生じることが分かった[11]．Fig. 5 は，PV100 の構造を示している．PV100 は，N 末端のシグナルペプチドの後に，システイン残基に富む 11 kDa の C ドメイン，アルギニンとグルタミン酸に富む 34 kDa の RE ドメイン，そして最後に 50 kDa のビシリン様領域からなっている．C ドメイ

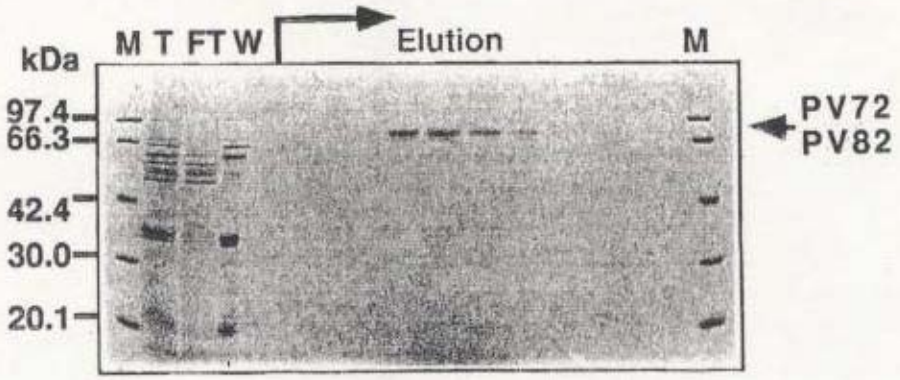

Fig. 3. Affinity chromatographic analysis of PV72/82 showing binding to a vacuolar targeting sequence of barley aleurain (proaleurain peptide, PAP). CHAPS extract containing the microsome membrane was applied to the proaleurain column. After washing with CHAPS buffer containing 500 mM NaCl, bound proteins were eluted by the addition of the same peptide in CHAPS buffer. Each of CHAPS extract (T), unbound fraction (FT) and wash fraction (W), and each eluted fraction were subjected to SDS-PAGE followed by CBB staining. M, molecular mass markers. Both PV72 and PV82 are indicated by an arrow.

Peptide	Sequence	Binding
2S-N	YRTTITIVEVEENRQGRE	-
2S-I	SRDVLQMRGIENPWRREG	+
2S-I/4G	----------GGGG---	+
2S-I/3G	----------GGG-	-
2S-C	KARNLPSMCGIRPQRCDF	+
2S-C/4G	---GGGG----------	-
PAP	SSSSFADSNPIRPVTDRAASTY	+

Fig. 4. Essential elements in the 2S albumin propeptides for PV72/82 binding. An internal propeptide (2S-I) and C-terminal peptide (2S-C) of pro2S albumin can bind to PV72/82, as well as a propeptide of barley aleurain. An NPIR sequence has been shown to be a vacuolar targeting signal of aleurain. We substituted the NPWR of the 2S-I peptide with GGGG to produce the 2S-I/4G peptide and prepared a 2S-I/4G affinity column. Unexpectedly, PV72/82 specifically bound to the mutated peptide. When the RRE sequence of the 2S-I peptide was substituted with GGG to produce the 2S-I/3G peptide, PV72/82 could not bind to the mutated peptide. We also substituted the NLPS of the 2S-C peptide with GGGG to produce a 2S-C/4G peptide. The NLPS sequence was essential for PV72/82 binding to the 2S-C peptide.

ンやREドメインは，種子内ではさらにプロセスされることが分かった．Fig. 5には，液胞プロセシング酵素がプロセスする部位と，それによって生じるたん白質を図示している．この図からも分かるとおり，PV100の一次構造上にはNQ（アスパラギン－グルタミン）配列が多数存在する．アスパラギンに対して特異性を持つ液胞プロセシング酵素はこの配列を認識して，アスパラギン残基のカルボニル基側を切断する[23,24]．その結果生じたN末端のグルタミン残基は，液胞内の酸性条件下でピログルタミン酸に環化する．N末端にピログルタミン酸を持つ低分子量のたん白質は液胞内に存在するアミノペプチダーゼなどの酵素に対して抵抗性を持つため，分解されずに存在するものと思われる．

ビシリンは主要な種子たん白質として多くの種子にみられるが，PV100のような前駆体構造を持つものはこれまでに知られていない．PV100のように一つの前駆体たん白質から複数の種子たん白質を生じさせる液胞内のプロセシングの機構は非常に面白い．

液胞プロセシング酵素は，種子の貯蔵たん白質の成熟化に関与する酵素として見出された酵素であるが，栄養器官型のホモログが最近新たに見つかってきた[25]．栄養器官における分解型液胞においても，たん白質蓄積型液胞と同様のプロセシング系が機能していると考えられる．

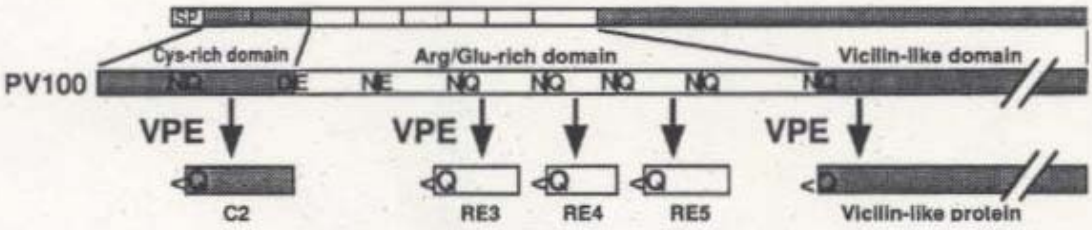

Fig. 5. A hypothetical mechanism for the VPE-mediated cleavage at Asn-Gln bonds to produce multiple seed proteins. PV100 precursor was composed of a hydrophobic signal peptide and the following three domains: an 11-kDa Cys-rich domain with four CxxxC motifs (C, Cys), a 34-kDa Arg/Glu-rich domain composed of six homologous repeats, and a 50-kDa vicilin-like domain. Two Cys-rich peptides, three Arg/Glu-rich peptides and the vicilin-like protein were produced by cleaving Asn-Gln bonds of PV100 and that all these proteins had a pyroglutamate at their NH_2 terminus. Vacuolar processing enzyme (VPE) is responsible for maturation of multiple seed proteins by cleaving Asn-Gln bonds of the PV100.

要　約

種子貯蔵たん白質は細胞内の粗面小胞体で合成された後に，たん白質蓄積型液胞に輸送され，蓄積される．液胞への貯蔵たん白質の選別輸送に関わるPAC小胞の解析から，膜たん白質PV72/82が主要な貯蔵たん白質2Sアルブミンのプロペプチドと特異的に結合することが分かった．即ち，PV72/82は2Sアルブミンの選別輸送レセプターとして機能していることが強く示唆された．ついで，PAC小胞の主要構成成分の一つPV100の構造解析から，液胞内のプロセシングの新規の機構が明らかになった．即ち，PV100は，一次構造上にAsn-Gln配列を複数持つが，この部位でプロセスされることにより複数の種子たん白質を生じる．これらの種子たん白質はいずれもN末端にピログルタミン酸を有することが分かった．

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