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Plant Seed Cystatins and Their Target Enzymes of Endogenous and Exogenous Origin

SOICHI ARAI,† ICHIRO MATSUMOTO,‡ YASUFUMI EMORI,§ AND KEIKO ABE*,‡

Department of Nutritional Science, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-0054, Japan; Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; and Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Cystatins are protein inhibitors of cysteine proteinases of the papain family, and those of animal origin have long been studied from medical and physiological aspects. In the meantime, oryzacystatin cloned from rice seeds in 1987 was recognized as the first well-defined cystatin of plant origin. Cloning studies followed to disclose various plant cytstatins including those of corn and soybean origin, their similarities to and differences from animal cystatins being analyzed in detail. Plant seed cystatins are now understood as factors controlling germination by inhibition of endogenous cysteine proteinases. They can also recognize insect midgut proteinases as exogenous target enzymes to control. This paper discusses chemical and phytophysiological relationships between cystatins and their targets.

KEYWORDS: Cystatins; protein inhibitors; cysteine proteinases; papain; oryzacystatin; target enzymes

There is historical background describing events that led to the initiation of studies on plant seed cystatins in Japan. During the 1980s, a Tokyo group extensively investigated a papain-catalyzed reverse reaction, a new version of the conventional plastein reaction, for the covalent incorporation of nutritionally essential or physically interesting amino acids into food proteins (*1*). This new reaction is characterized by aminolysis that facilitates the efficient incorporation of amino acids (ester forms) as nucleophiles by attacking the peptidyl−papain intermediate. To control this reaction, the Tokyo group needed a papain inhibitor that could be warranted as safe for food processing. With this as background, another Tokyo group chose rice as a well-qualified source that might contain an inhibitor of cysteine proteinases including papain. Thus, the group successfully found a cystatin named oryzacystatin (2), which can be classified as a protein inhibitor of cysteine proteinases belonging only to the papain family, not to the calpain family. Oryzacystatin was then internationally recognized as the first well-defined cystatin of plant origin (*3*).

A great many studies have been conducted on cystatins of animal origin, primarily in the fields of medical and physiological sciences. The chemical structures and physiological properties of a large number of cystatins occurring in animal tissues and products have been elucidated. According to these data, an international ad hoc committee attempted to classify animal cystatins into three families (*4*). Family 1 is sometimes called the stefin family. It comprises single-chain proteins that lack disulfide bonds, with molecular weights of ∼11000. Family 1 cystatins are also characterized by their close sequence similarities to one another, especially in terms of the conserved Gln-Val-Val-Ala-Gly loop existing in a central region of each molecule. Family 2 is called the cystatin family in a broad sense, composed of single-chain proteins with molecular weights of ∼15000, each of which has two intramolecular disulfide bonds. They also share high sequence similarities, but their central pentapeptide site is in the form of Gln-Xaa-Val-Xaa-Gly. Family 3 cystatins are exclusively the much higher molecular weight proteins, plasma kininogens, as the precursors of vasoactive kinins and also as those that participate in the blood coagulation cascade. Therefore, this family is often called the kininogen family. It is characteristic that each kininogen protein molecule contains repeats of a family 1 cystatin-like sequence with Gln-Val-Val-Ala-Gly. Thus, these three families were unified into the cystatin superfamily (*4*). However, the classification and unification made at that time were based only on the knowledge of animal cystatins because no crucial information on plant cystatins was available.

ORYZACYSTATINS AND OTHER PLANT SEED CYSTATINS

As mentioned above (*1*), our need for a safe substance to be used to control the plastein reaction motivated us to exploit a papain inhibitor from rice, a staple food consumed through the entire history of Japan. We successfully found such an inhibitor in ripening seeds of rice, *Oryza sativa* L. *japonica*, and characterized it first at the protein level and then at the DNA

* Author to whom correspondence should be addressed (telephone +81-3-5841-5129; fax +81-3-5841-8006; e-mail aka7308@mail.ecc.u-tokyo.ac.jp).
† Tokyo University of Agriculture.
‡ Department of Applied Biological Chemistry, The University of Tokyo.
§ Department of Biophysics and Biochemistry, The University of Tokyo.

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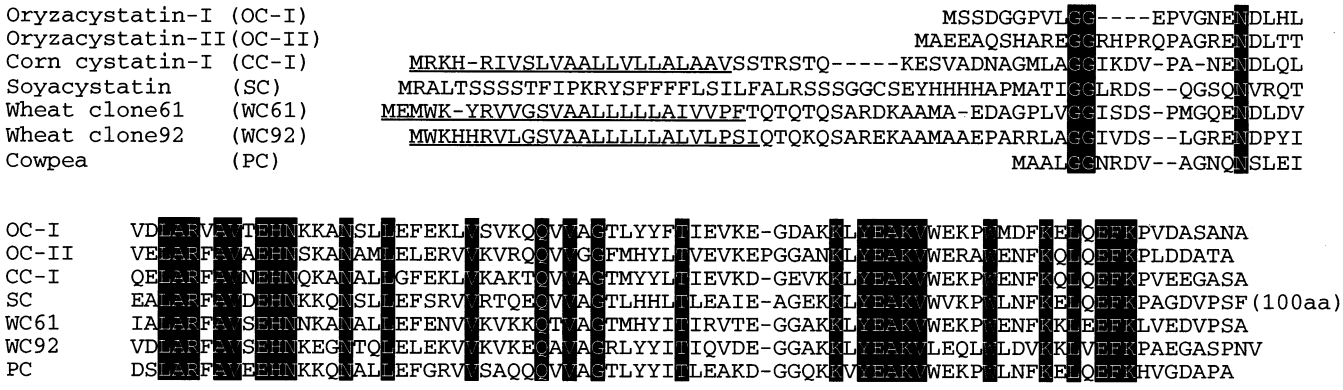

Figure 1. Amino acid sequences of plant seed cystatins as representative phytocystatins: OC-I, oryzacystatin I; OC-II, oryzacystatin II; CC-I, corn cystatin I; CC-II, corn cystatin II; WC, wheat cystatin 1; SC, soyacystatin; WSC, wound-inducible soybean cystatin; COC, cowpea cystatin; CAC, carrot cystatin; SFC, sunflower cystatin. Three consensus sites are indicated. White letters on black denote commonly conserved amino acid residues.

level (*2*). The inhibitor occurs at ~2−3 mg/kg of seed. The purified preparation inhibits papain efficiently ($K_i \sim 10^{-8}$ M) and is characterized by its heat stability. Even after treatment at 100 °C for 30 min (cooking conditions), it retains most of its papain inhibitory activity. This means that people have long taken an active form of the inhibitor on a daily basis with no deleterious health problem.

For molecular cloning, we prepared a λgt10 phage cDNA library of immature rice seeds and screened it with two oligonucleotide probes synthesized according to the partial amino acid sequences Lys-Pro-Trp-Met-Asp-Phe and Lys-Pro-Val-Asp-Ser of the inhibitors. A full-length cDNA clone encoding 102 amino acid residues was thus obtained, and the primary structure deduced from its nucleotide sequence was found to be homologous with those of family 2 cystatins. It conserves the central pentapeptide sequence, Gln-Val-Val-Ala-Gly, which is now recognized as the target enzyme-binding site. This is why the papain inhibitor in rice seeds was named oryzacystatin (*2*). Subsequently, we isolated another oryzacystatin cDNA clone by screening with the first oryzacystatin cDNA as a probe (*5*). The two oryzacystatin species were then renamed oryzacystatins I and II in the order of discovery. Oryzacystatin II showed 55% sequence similarity with oryzacystatin I and contained the target enzyme-binding site in the form of Gln-Val-Val-Gly-Gly instead of Gln-Val-Val-Ala-Gly. Interestingly, both oryzacystatins are structural chimers of family 1 and family 2 cystatins, in that each of them lacks disulfide bonds regardless of their resemblance to family 2 cystatins with respect to amino acid sequence. The same is true for other plant cystatins found by us and by independent groups.

We investigated other monocotyledonous plants, corn and wheat, and dicotyledonous plant, soybean, to find new cystatins. Two corn cystatins, I and II, were identified in immature corn kernels (*6*, *7*). Both are apparently members of the cystatin superfamily, because they have a Gln-Xaa-Val-Xaa-Gly loop in their central regions. Bacterially expressed corn cystatins I and II showed effective papain inhibitory activity, and corn cystatin II inhibits cathepsin L rather than papain. Seeds of wheat express several cystatin mRNAs. Recombinantly produced wheat cystatins 1 and 4 inhibit cathepsin B to some extent ($K_i \sim 10^{-6}$ M) and cathepsins L and H to a greater extent ($K_i \sim 10^{-8}-10^{-9}$ M). Also, each of the three wheat cystatins effectively inhibits cysteine proteases, which occur in wheat seeds (unpublished data). Soyacystatin, which we identified in dicotyledonous soybean, has some striking structural features at amino acid and nucleotide levels (*8*).

Subsequently, other groups working independently have initiated plant cystatin studies and found cystatins in potato (*9*), ragweed (*10*), avocado (*11*), papaya (*12*), apple (*13*), herbs (*14*), etc., as well as in the seeds of cowpea (*15*), carrot (*16*), sunflower (*17*), and wound soybean (*18*). **Figure 1** shows representatives of known plant seed cystatins aligned to compare their amino acid sequences (**Figure 1**).

Recently, an attempt was made to add new members to the cystatin superfamily. A good example is offered by fetuins, which were first characterized in 1944 (*19*) and found in 1988 to be related to cystatins (*20*). Another example may be a histidine-rich glycoprotein that generally occurs in the plasma of mammals (*21*) and is structurally related to high molecular weight kininogen. Gene structure analyses showed that the human histidine-rich glycoprotein has cystatin domains that are each encoded by three exons as they are in many cystatins. Meanwhile, variant cystatins were purified and characterized. These include a protein occurring in the venom of African puff adder (*22*) and also proteins isolated from fruit flies, *Drosophila melanogaster* (*23*). The existence of divergent cystatin in *D. melanogaster* is interesting because we have cloned its cysteine proteinases as described later. In considering all of the cystatin superfamily members including these new entries, it is noteworthy that all of the plant seed cystatins we found to lack disulfide bonds, perhaps making it appropriate to include them in the plant stefin or phytocystatin family. Along these lines, a recent interesting paper (*24*) reports a multialignment and a phylogenetic analysis of 63 cystatins, 32 of which are plant cystatins, including oryzacystatins. The results demonstrate that all plant cystatins cluster in a major evolutionary tree branch and support their classification as a new cystatin family. The appropriateness of the "phytocystatin family" we proposed (*3*) has thus been reconfirmed.

ENDOGENOUS TARGET ENZYMES

Most plant seed cystatins have been investigated for developmental stage and tissue distribution of their expression. Oryzacystatins I and II are synthesized in rice seeds during maturation. They occur in the cytosol and are decomposed as soon as germination starts (*2*, *5*). A similar phenomenon is observed for corn cystatins, although each has a signal peptide suggesting that it may be a vacuole-secretory protein (*6*, *7*). For soyacystatin, the observed event is that its mRNA is expressed in seeds two weeks after flowering and that it is expressed nearly uniformly in the cotyledons (*8*). These

phytophysiological events suggest the involvement of some cysteine proteinases targeted by seed cystatins.

Meanwhile, close attention has been given to aleurain, a barley cysteine proteinase the expression of which is induced by gibberellin (*25*). This encouraged us to investigate the gibberellin-aided expression of cysteine proteinases in rice grains, where we found them to be possible target enzymes of oryzacystatin. Screening a cDNA library constructed from germinating rice seed with aleurain cDNA as a probe yielded three independent clones, each encoding a protein having a catalytic triad (*26*) and resembling papain as well as aleurain. The deduced amino acid sequences of the three enzymes, named oryzains α, β, and γ, were obtained (*27*). Homology search and consequent genealogy gave a pedigree showing that oryzains α and β are closely related to papain, whereas oryzain γ most resembles cathepsin H (**Figure 2**). Considering the inhibition spectra of oryzacystatins against papain and cathepsin H, it is possible that oryzains α and β are endogenous target enzymes of oryzacystatin I, whereas oryzain γ is targeted by oryzacystatin II.

Oryzains α and β are highly homologous to each other but differ significantly in developmental stage of their expression. Oryzain α mRNA is detected only during germination, with a maximum level reached 5 days after the start of germination. On the other hand, oryzain β mRNA already exists even in mature seeds when no oryzain α mRNA is detected and begins to increase remarkably upon germination. As expected, the expression of these mRNA species and that of oryzains α and β themselves are induced by gibberellin in vitro. In the presence of added gibberellic acid (GA3) at the proper concentration in the medium, dipped rice seeds express oryzain α mRNA and oryzain β mRNA, the quantities of which reached plateaus within 1 day and within as little as 4 h, respectively. This is consistent with the result that the expression of oryzain β mRNA is followed by that of oryzain α mRNA. Immunohistochemical analyses revealed that oryzain α exists in both the aleurone and the endosperm, whereas oryzain β localizes only in the aleurone in early germinating seeds (unpublished data). These observations suggest that oryzain β is involved in early event(s) in germination such as primary digestion of storage proteins and processing of sequentially expressed proteinase(s) including oryzain α and that oryzain α is involved in later events in germination such as proteolysis of storage proteins to free amino acids.

Recently, our group found several cereal cysteine proteinases including two in corn kernels (*29*) and four in wheat grains (unpublished data). Independent groups have also verified the occurrence of new cysteine proteinases and their possible phytophysiological functions. Some plant cystatins probably control the activities of these cysteine proteinases in tissue-specific and/or developmental stage-specific fashions. In addition, some may also be involved in more nonspecific control. Plant cystatins can probably recognize plant cysteine proteinases as endogenous target enzymes to control their activities.

Very recently, an extremely interesting paper was presented reporting the involvement of soybean cysteine proteinases and cystatins in the control of programmed cell death, that is, apotopsis (*18*). As cysteine proteinases emerge as key enzymes in animal apotopsis, in soybean cells as well it has been shown that apotopsis-activating oxidative stress induces a set of cysteine proteinases. Thus, their inhibition by the ectopic expression of cystatin could control the induced enzyme activity and block the apotopsis triggered by oxidative stress. This result adds a new dimension to the study of the functions of plant seed cystatins.

EXOGENOUS TARGET ENZYMES

Plant seed cystatins may have exogenous target enzymes as well, which often originate from invading viruses, bacteria, and insects. In recent years, the significance of these cystatins in controlling cysteine proteinases of insect origin has been evaluated, and thus the importance of investigating those existing in the digestive tract has been especially stressed. However, it was not until 1995 that our group first investigated insect gut cysteine proteinases at the molecular level in order to find out how plant seeds defend themselves against pests (*30*). To elucidate a molecular mechanism for this biodefense phenomenon, we isolated a gene encoding a putative digestive cysteine proteinase from *D. melanogaster*, a suitable model species. The amino acid sequence of this enzyme also showed significant similarities to cysteine proteinases of animal origin, such as cathepsins H and L, and to proteinases of plant origin, such as rice oryzains α and β. In situ hybridization studies of the embryo showed that the mRNA for *Drosophila* cysteine proteinase 1 is expressed predominantly in the midgut. Larval alimentary organs, such as the salivary gland and the midgut including the gastric caeca, also express the mRNA at significant levels. It is hoped that these observations, suggesting that *Drosophila* cysteine proteinase 1 is a digestive cysteine proteinase that can be used as a model target of phytocystatins, will lead to new strategies for the control of pest insects.

Subsequently, we identified and characterized a gene family comprising at least four genes encoding cathepsin L-like cysteine proteinases in *Sitophilus zeamais*, Coleoptera (*31*). These *Sitophilus* cysteine proteinases show high sequence similarities to one another as well as to other insect and mammalian cathepsin L-like proteinases (**Figure 3**). A polyclonal antibody raised against a bacterially expressed preparation was used as a probe to examine the molecular forms and distribution of the enzyme. The enzyme exists in both proenzyme and mature forms in larvae, pupae, and adults, and the proenzyme is converted in vitro into the mature form at acidic pH. Immunohistochemical analysis showed that the enzyme is present in several tissues including alimentary organs and germ cells. In alimentary organs, it is distributed in the gastric cecum, but not in the midgut. It is also present in genital organs, especially in oocytes and nurse cells, where it exists at high levels. These results indicate that this enzyme plays a variety of physiological roles including a role in food digestion.

To characterize in more detail the cathepsin L-like cysteine proteinases from *S. zeamais* (SCPs) cloned, we established a system for their functional expression and purification using a glutathione *S*-transferase (GST) fusion gene vector from *Escherichia coli* (*32*). The proenzyme forms of two representative SCPs, proSCPc1 and proSCPg3, were expressed as GST fusion proteins and purified on a glutathione Sepharose column. GST−proSCPc1 undergoes autoproteolytic cleavage to the mature enzyme efficiently at acidic pH and exhibits significant proteolytic activity toward various substrates including hemoglobin and Z-Phe-Arg-MCA. The enzymatic characteristics of the activated form of SCPc1 are similar to those of mammalian cathepsin L, but its pH optimum for the hydrolysis of hemoglobin is significantly lower. This suggests SCPc1 could be a target of phytocystatins, especially oryzacystatin I. As expected, proteolytic activity of SCPc1 was inhibited by oryzacystatin I as well as egg white cystatin and E-64 (*32*). This indicates the potential of phytocystatins as bioprotectant agents. However, plant seeds having cystatins as a defensive factor are fed on by insects having cysteine proteinase as a digestive enzyme. To address this paradoxical event occurring in nature, a bioassay

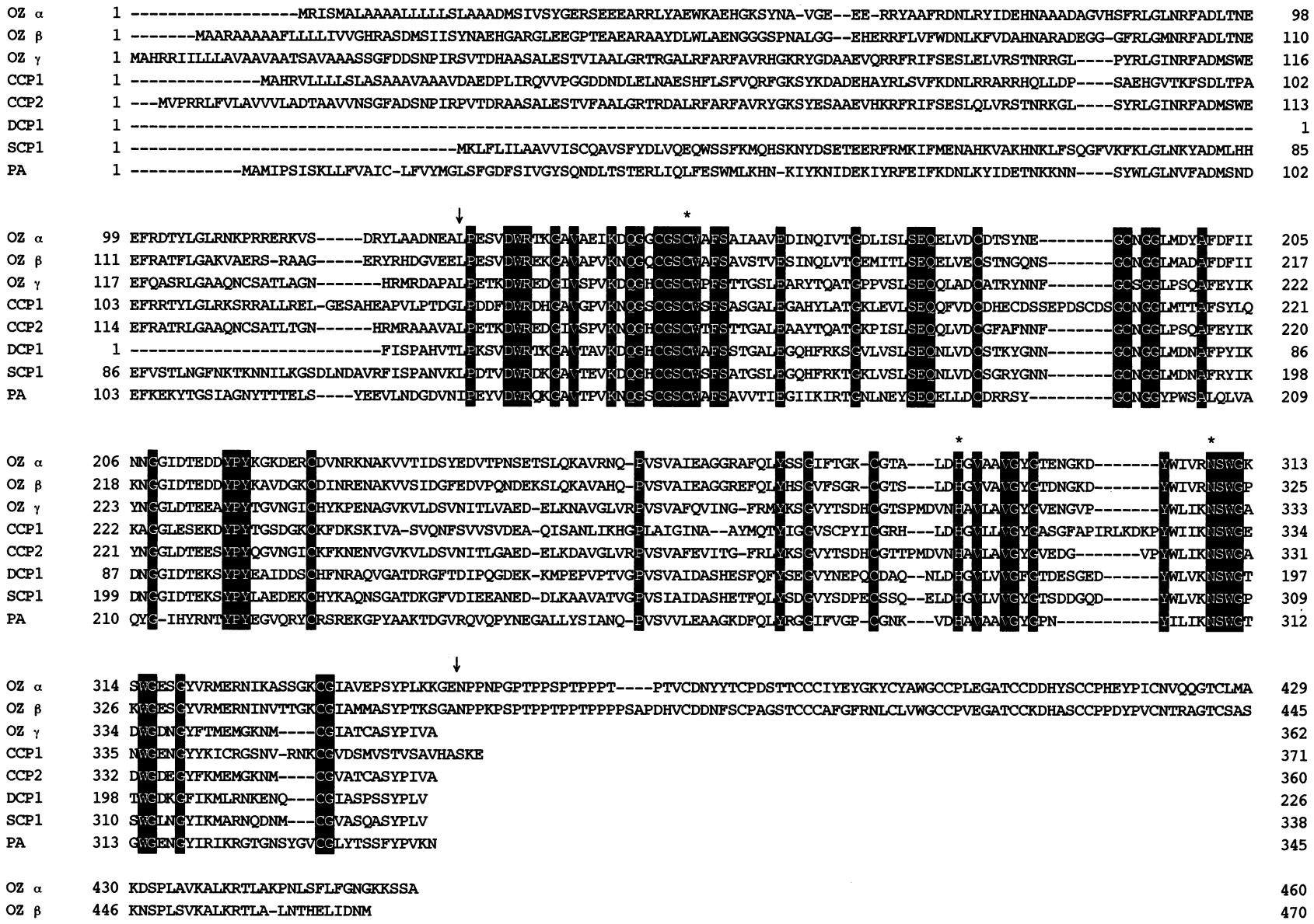

Figure 2. Amino acid sequences of oryzain and other new cysteine proteinases aligned with papain as a reference: OZ α, oryzain α; OZ β, oryzain β; OZ γ, oryzain γ; CCP2, corn cysteine proteinase 2; DCP1, *Drosophila* cysteine proteinase 1; SCP1, *Sitophilus* cysteine proteinase 1; PA, papain as a reference with the catalytic triad Cys-25, His-159, Asn-175 asterisked. Arrows point out possible scissile bonds for the N and C termini of mature enzymes. White letters on black denote commonly conserved amino acid residues.

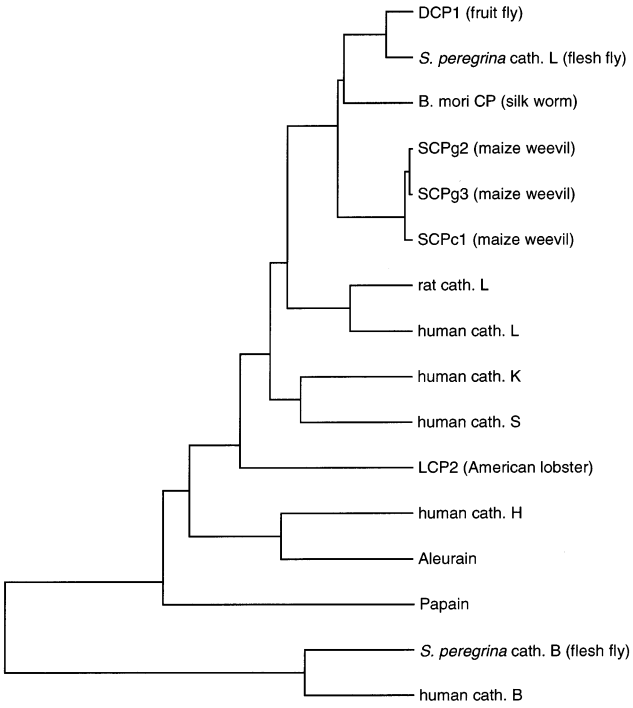

Figure 3. Phylogenetic tree of representative cysteine proteinases (CPs) including insect CPs.

was carried out. The addition of oryzacystatin I to the diet exerts significant effects against several insects, for example, maize weevil. Even a lower concentration of added oryzacystatin I causes growth retardation, and at a higher concentration, lethality results. Thus, insect pests may have digestive cysteine proteinases in amounts much larger than those of cystatins in the plant seeds they eat. Simultaneously, this suggests the usefulness of phytocystatins for insect pest control. Kuroda et al. (*33*) have observed the insect-controlling effects of recombinant oryzacystatins, reporting that bacterially produced OC-I and OC-II cause growth retardation of different species of bean insect pests, *Callosobruchus chinensis* (Coleoptera) and *Riptortus clavatus* (Hemiptera), when added to their diets at concentrations of 0.3−0.5% (w/w). At higher concentrations, nearly all insects died. These results suggest again the usefulness of cystatins for insect pest control and also the critical role of cysteine proteinases in digestive events in insects.

CONCLUSION

Considering all of the data we have obtained on multiple species of cystatins in rice, corn, wheat, and soybean, it is possible that these inhibitors occur universally in plants, regulating their intracellular protein catabolism. This possibility must be high, as it has been found that these plants contain endogenous enzymes that can be almost stoichiometrically inhibited by the respective cystatins. Also, plants as immobile organisms per se have a variety of parasite-defense systems, and it is likely that the seed cystatins are involved in such systems. Further research into such regulatory and defensive mechanisms will faciliate the development of transgenic crops with enhanced oryzacystatin and the like for agricultural benefit, as documented by a number of recent papers (*34*−*39*).

For particular information about the usefulness of cysteine proteinase inhibitors, including cystatins, in controlling insect pests, see our review, which also discusses the significance of constructing transgenic plants with enhanced cystatin levels (*40*). A reference is added to show a three-dimensional NMR structure of oryzacyctatin I, which elicites a strong antipest activity (*41*).

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