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FUNCTIONS OF INHIBITORS OF PROTEOLYTIC ENZYMES IN PLANTS

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The literature on proteinase inhibitors of plant origin over the last 15 years is discussed. The results of investigations are permitting an expansion of our ideas on the regulatory and protective functions of proteinase inhibitors, which play a primary role in the resistance of plants to phytopathogens and can be used as a test in selection practice.

A group of proteins inhibiting proteolytic enzymes is widely distributed in plants. They were discovered more than 50 years ago and have been studied particularly intensively in the last 15-20 years [1-4].

The practical interest in protein inhibitors of proteolytic enzymes of animals, plants, and microorganisms is due to their wide use in biochemical investigations and biotechnology, and also in medical practice, including the treatment of oncological diseases and AIDS [5-9]. The particular interest in plant proteinase inhibitors as resistance factors is due at the present time to the possibility of finding and enhancing protective systems both with the use of traditional selection methods and on the basis of the genes of proteinase inhibitors ensuring prolonged resistance [10, 11].

Inhibitors of all known classes of proteinases and, in the first place, proteinases of the serine type — trypsin and chymotrypsin — are found in representatives of many families and species of plants, especially among the Leguminosae. An analysis of the literature on the distribution, classification, localization, and the comparative structural investigation of proteinase inhibitors and also on their functions in plants has been given in fairly great detail in a review by V. V. Mosolov and T. A. Valueva [1]. The results of their investigations and information in the literature show that the main function of proteinase inhibitors in plants consists in the regulation of the hydrolysis of reserve proteins by proteolytic enzymes. They may fulfil the function of protective agents against serine proteinases of various types of phytopathogens and insects. The considerable level of inhibitors in the reserve organs of plants may be connected with their possible function as reserve proteins.

In the present paper, our main attention is devoted to two important functional properties of plant proteinase inhibitors — their regulatory role in the system of proteolysis of the reserve proteins, and their protective role, ensuring the resistance of the plant to harmful factors. The possibility has been shown of testing the degree of resistance of plants in selection breeding, including the creation of promising varieties of the cotton plant.

Regulatory Function of Proteinase Inhibitors

In recent years it has been shown for a number of plants, as examples, that proteinase inhibitors interact with the corresponding proteolytic enzymes, exhibiting, in so doing, an exceptionally high specificity and fulfilling a definite role in the regulation of the proteolysis of the reserve proteins of seeds in dormancy and on germination [1, 12-14].

A possible basic mechanism of the regulation of the activity of endogenous proteinases is the capacity of inhibitor proteins for forming stable inactive complexes with the enzymes. On the germination of the seeds, usually in the course of the first three days, the complex breaks down, as is shown by a fall in the activity of trypsin inhibitors and a rise in the activity of proteinases [1, 15, 16].

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One of the main processes in the germination of plant seeds is hydrolysis of the reserve proteins, the products of which are used for forming the developing seedling. And here the existence of a regulatory system including a reserve substance and a hydrolase is suggested. The necessity for smooth regulation is due primarily to the metabolic requirements of the growing axial organs. Bioregulation is achieved by the feedback principle; i.e., the end-products of breakdown inhibit the whole chain of reactions responsible for the complete hydrolysis of the reserve substances. With a constant efflux of the end-products of breakdown into the actively growing part of the seedling a rapid breakdown of the reserve substances takes place. When growth processes in the seeds are inhibited, the end-products of breakdown accumulate, and this leads to a retardation of hydrolysis. Consequently, the presence in the seeds of high-molecular-mass reserve substances and of enzymes splitting them permits a regulated feed of physiologically active metabolites — in this case, amino acids and peptides — to the growing part of the seedling [16].

On the other hand, as already mentioned, the mechanism of the regulation of the action of proteolytic enzymes presupposes the existence of a proteinase–inhibitor system. The regulatory role of proteinase inhibitors in the growth of a number of plants has been considered in several publications [1, 5, 17-19], A general law has been revealed that consists in a decrease in the activity of the proteinase inhibitors during the growth of barley, maize, pea, and other, seeds. One of the causes of the degradation of trypsin inhibitors during the germination of, for example, lupin seeds is the cleavage of the inhibitors by cysteine proteinases [20].

The existence of a more complex "proteinase–inhibitor–substrate" system of regulation was first shown by M. A. Belozerskii et al. for the case of buckwheat seeds [21-23]. They succeeded in extracting protein bodies from the seeds and showing the simultaneous presence in them of a metalloproteinase and its inhibitor, localized within the cell together with reserve proteins. This confirmed the probability of the above-mentioned complex system of proteolysis regulation that is necessary during the maturation of the seeds for preventing the breakdown of the reserve proteins, as shown for the case of the formation of a protease–inhibitor complex in lupin seeds [15].

The existence of a system for the regulation of the proteolysis of reserve proteins, including proteinase inhibitors, may also be suggested on the basis of a comparison of results that we have obtained on the change in the activity of proteinase inhibitors in germinating cotton seeds.

On investigating the activity of proteinase A, of the serine type, and its protein inhibitor at various stages of the germination of cotton seeds in the course of three days, we found that a fall in the activity of the inhibitor correlated with a rise in the activity of the proteinase A [24]. Simultaneously, we studied the proteolysis of the main reserve protein — the 11S globulin — at the beginning of germination. The correlation in the change in the activities of proteinase A and its inhibitor during germination and the capacity of proteinase A for hydrolyzing the corresponding reserve protein permits the assumption of the existence of a proteinase–inhibitor system including the reserve protein of the cotton plant.

Thus, in plants a complex system for the regulation of the proteolysis of reserve proteins exists that includes protein inhibitors of proteinases as one of the active participants in this process.

Role of Proteinase Inhibitors in Plant Protection

Investigations of recent years have shown that plants possess a system of protective reactions that are activated in response to phytopathogenic infection, on attack by insects, or under the action of other types of stress. Such protective mechanisms include not only the synthesis and accumulation of substances of the type of phytoalexins but also an increase in the activity of a number of protein substances [25]. The latter include a number of plant enzymes [26], lectins [27, 28], polypeptide toxins of the thionin class [29, 30], and protein proteinase inhibitors [1, 10, 31, 32].

No interaction of the inhibitors with a harmful factor has been shown at the molecular level, but their capacity for suppressing the proteolytic activity of the proteinases of the digestive tract of insect pests has been demonstrated. Such results have been obtained for inhibitors from soybeans, rice, chick peas, cow peas, amaranth, and wheat [1, 33-36].

Many inhibitors exert an analogous action on the proteinases of phytopathogenic organisms [37, 38]. Thus, proteinase inhibitors, predominantly of the serine type, from kidney bean seeds depressed the activity of the proteinases of a *Fusarium* fungus and suppressed the growth of this fungus on an artificial nutrient medium. Trypsin and chymotrypsin inhibitors from dormant buckwheat seeds inhibited the growth and development of the mycelium of *Alternaria tenius* Nees [39]. The same results were obtained in a study of the action of proteinase inhibitors isolated from lupin seeds on the activity of the proteases of *Fusarium oxysporum* [40]. Moreover, comparative evaluations of the activities of proteinase inhibitors of varieties of lupin and potato susceptible and resistant to diseases have revealed a correlation between inhibitor activity and the resistance of the plants to phytopathogenic infection [41]. A similar relationship has been detected between the activity of a trypsin inhibitor and the resistance of individual varieties of wheat to fungal diseases [42].

The results of our investigations have shown that protein inhibitors isolated from dormant cotton seeds depress the activity of the proteolytic enzymes of the fungus *Verticillium dahliae* and suppress the growth of its mycelium on Czapek-Dox medium [43-46]. At the same time, we observed different degrees of suppression of the growth of the fungus by inhibitors from healthy and diseased cotton seeds, since the affected seeds contained a higher level of inhibitors than the healthy seeds.

The toxic effect of protein proteinase inhibitors in relation to the physiology of the digestive process of insects, and also the induction of the synthesis of inhibitors in response to damage to the plant by phytopathogens has served as a basis for including inhibitors among the most important factors of plant resistance. Here we specially consider inhibitors of serine and cysteine proteinases, and also those of aspartyl and metalloproteinases [10].

Recently, in connection with the revelation of a positive correlative interaction between the level of proteainse inhibitors and the resistance of plants to phytopathogenic infection, the possibility has appeared of using a new approach in selection studies in which proteinase inhibitors can serve as markers of plant resistance.

An effective method of evaluating potatoes for resistance to *Fusarium sambucinum*, based on determining the relationship between the activity of trypsin inhibitors and the susceptibility of the tubers, has been proposed by N. A. Dorozhkin et al. [47]. The high degree of correlation has permitted the development of a simple method for evaluating the resistance of the potato to the phytopathogen.

We have developed a highly sensitive immunochemical method of determining wilt resistance that is based on the highly specific interaction of antibodies with the antigen to be determined and have employed this method of analysis for the quantitative determination of a proteinase A inhibitor in wilt-resistant and susceptible varieties of cotton. The result of the ring-precipitation reactions that we performed showed a linear relationship between the concentration of antigen (proteinase A inhibitor) and wilt resistance [43].

Feinberg's more sensitive concentration gradient method is also available. By using this method, we have determined the dependence of the resistance of a cotton plant to wilt on the amount of inhibitors synthesized in it on its being damaged. This has permitted a quantitative evaluation of the degree of resistance of a number of new selection varieties of the cotton plant and an acceleration of selection studies.

However, the available information about the action of proteinase inhibitors on pathogenic microorganisms does not reflect their specific roles in the interrelationship of pathogen and host plant. In view of this, considerable interest is presented by a study of the proteinase–inhibitor system in plant tissues during the process of pathogenesis. B. B. Gromova was the first in phytopathological practice to investigate in detail the question of parasitism and the nature of plant resistance from the aspect of the immunochemical similarity of the antigens of the pathogen and of the plant [48]. This showed that the level of proteinase inhibitors depends on the degree of resistance of the potato to infection by a virus. Analogous properties, enabling the resistance of a plant to be tested from the accumulation of trypsin inhibitor, have been revealed for rice, barley, and other ceral crops [2, 49].

The study of the level of activity of trypsin-like enzymes and their inhibitors during the maturation of seeds in varieties of wheat resistant and susceptible to infection by loose smut has shown considerable differences in their response reaction to the introduction of the pathogen. Plants of a resistant variety are characterized by a high level of the activity of proteinase inhibitors at all stages of the maturation of the seeds. The dynamics of the accumulation of proteinase inhibitors in the seeds of a susceptible variety shows the absence of their accumulation in response to affection. Considerable differences in the activity of proteinase inhibitors have also been detected between resistant varieties and in dormant wheat seeds [49]. We have also found fundamental differences in the level of inhibitors in cotton seeds from healthy and infected plants of two varieties contrasted with respect to their degrees of resistance to verticillium wilt. In the resistant variety, the level of the inhibitor fraction was 1.7 times higher than in the susceptible variety [50]. Furthermore, under the influence of wilt infection the level of inhibitors rose somewhat only in the resistant variety, while in the susceptible variety it remained unchanged.

A rise in the level of inhibitors in a plant in response to affection by phytopathogens or insect pests shows an active participation of proteinase inhibitors in the immune response of the plant. As has been stated above, the mechanism of these processes has been studied inadequately, although suggestions exist that the response reaction of a plant is connected with the formation either of a "wound hormone" or of definite receptor proteins produced as the result of damage to the cell walls of plants and fungi, inducing the biosynthesis of proteinase inhibitors [1].

Together with the proteinase inhibitors and the other protein compounds mentioned above that participate in the system of plant-protective reactions, a new class of proteins possessing fungicidal properties has recently been discovered [51, 52]. They have been assigned to the 2S reserve proteins and in the course of structural investigations have shown a high degree of homology with proteinase inhibitors. The authors concerned suggest that the new class of proteins is present in a wide range of plants.

Thus, the study of plant resistance factors is in its initial stages. However, analysis of the literature of recent years permits us to characterize plant proteinase inhibitors as proteins taking part in the regulation of the proteolysis of reserve proteins and in the protective reactions of plants [1, 10, 53]. The results of a study of functional and structural features of proteinase inhibitors is permitting them to be used in practice as chemical tests for accelerating the selection breeding of resistant varieties of plants and also for gene-engineering methods of enhancing resistance characteristics. In addition, investigations of protein proteinase inhibitors are also important from the theoretical point of view for answering questions of the mechanism of the regulation of proteolysis and the immune status of a plant.

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