International Journal of Horticultural Science 2007, 13 (4): 35–40
Agroinform Publishing House, Budapest, Printed in Hungary
ISSN 1585-0404

Transmethylation and the general defense reaction of plants

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Summary: Plant breeding for resistance, namely building specific resistance genes into cultivated plants to ensure resistance against certain pathogen species, is a several-decade-long practice. While looking for purposes of failures appearing during the cultivation of varieties created in this way, a plant feature that ensures non-specific reactions against effects which evoke biotic stress attracted our attention. We named this plant defense form the *general defense reaction*. The general defense reaction is a fundamental attribute of the plant kingdom, fulfils the role of plant immune system and manifests itself in cell enlargement and cell division. Plants with a high level general defense reaction endure abiotic stresses as well.

In studying the biochemical background of the interaction of the general defense reaction and transmethylation, we found that transmethylation has important role in warding off both biotic and abiotic stresses. According to our observations, plants possessing high level general defense system are suitable for thorough examination of the process and plant physiological role of transmethylation. Biochemical studies also strengthened our observation, which has been taken on the basis of phenotype, that the general defense system can not be ignored during future plant breeding.

Key words: biotic and abiotic stress, general defense system (gene symbol: *gds*), OPLC, pepper (*Capsicum annuum*), transmethylation, watermelon (*Citrullus lanatus*)

Introduction

One of the most striking features of biological systems is considerable permanence of their internal balance. Through specific reactions, the internal balance is maintained and is restored in the case of its destabilization (*Bell*, 1981). If the influence of stress is so strong that it exceeds limits of a biological system, then permanent damage, exhaustion and cell death follows. If this effect does not reach this damaging value, temporary or permanent resistance may develop against subsequent stress effects. This may result from accumulation of substances which play a role in forming the ability to resist, in other words increasing the resistance potential, from the appearance of more efficient methods of defense, or from the collective effect of the aforementioned. Resistance develops through fast conversion reactions following the alarm reaction by means of endogenous substances which determine the resistance potential. Therefore we must turn our attention to compounds which play a part in these reactions. Such reactions may include reactions involved in methylation-demethylation and products of these reactions.

Transmethylation, the removal and addition of methyl groups, is a known substitution reaction. Enzymatic transmethylation reactions proceed indirectly through many steps, in which formaldehyde plays an important role. It has been proved that S-adenosyl-Lmethionine (SAM), which is an active form of L-methionine, is a methyl-donor in transmethylation reactions (*Paik & Kim*, 1980; *Szarvas* et al., 1986). It is also verified that the methyl group of SAM is transferred to the acceptor molecules (nuclein acids, proteins, peptides, amino acids) through formation of formaldehyde (HCHO), an intermediate product. This refers to the participation of HCHOin methylation reactions (*Huszti & Tyihák*, 1986).

It has been demonstrated that methyl groups of certain exogenous and endogenous N-, S-, O-methylated compounds can be regarded as precursors of HCHO in enzymatic demethylation reactions (*Szarvas* et al., 1986; *Kawata* et al., 1983). As a result of biotic and abiotic stresses, methylated compounds can be demethylated. HCHO molecules produced in this way can take part in reactions which will protect parts of a biological system that are sensitive to stress (*Sárdi & Tyihák*, 1991; *Kim* et al., 1992).

Biological transmethylation can be followed by simple analytical methods by means of measuring endogenous HCHO. Rising levels of HCHO signal the increasing rate of transfer of methyl groups, while falling levels are explained by the retention of methyl groups on HCHO generators.

Examination of different biological systems suggests that there is a strong correlation between the basic phases of stress syndrome (resistance potential, alarm reaction, development of resistance, normalization or exhaustion phase) (*Selye*, 1964) and transmethylation reactions.

Examinations of woody and annual plants suggest that qualitative and quantitative relationships between HCHO levels and certain quaternary ammonium compounds (N-trimethyl-L-lysine (TML), choline, carnitine, trigonelline, betaine) are connected with the resistance potential of a given plant. Accordingly these compounds play an important role in endogenous reactions induced by biogen and abiogen stress effects (*Tyihák* et al., 1978; *Sárdi & Tyihák*, 1992; *Sárdi*, 1994).

In the 21st century the number of biotic and abiotic stress factors affecting plants is increasing fast. Therefore it is a reasonable demand that besides reactions ensuring specific disease resistace, the general defense system (*Szarka & Csilléry*, 1995) of living organisms and its practical utility be examined in more detail. This is all the more important because so far in the history of plant breeding selection has not been made in the interest of the general defense system. On the contrary! Breeding work including resistance breeding, has, though not consciously, considerably ruined the general defense system of plants (*Szarka & Csilléry*, 2001a,b). General stress reactions of plants are completely independent of the specific resistance genes which ensure disease resistance. These genes are efficient only in the presence of a well-operating general defense system. Getting acquainted with biochemical processes may help to gain an exact understanding of general plant reactions which manifest themselves phenotypically. The energetic characterization of the general and the specific defense reaction of plants through glucose utilization, provided help in comparing these two processes. According to this, a low threshold limit and a high reaction speed is characteristic of the general defense reaction, while a higher threshold limit and a lower reaction speed is typical of the specific defense reaction (*Szarka* et al., 2002; *Csilléry* et al., 2004; *Sárdi* et al., 2006). These findings unambigously proved the role of these two defense reactions in plant defense, and that they are built on each other (*Szarka* et al., 2002; *Csilléry* et al., 2004).

The aim of our experiments is to investigate the role of transmethylation processes in the general defense reaction. In other words, whether qualitative and quantitative relationships between HCHO and certain quaternary ammonium compounds (which may be considered main formaldehyde generators) are suitable for characterization of defense reactions which manifest themselves in symptoms of a general nature.

Material and method

Plant material and infection

We worked with the susceptible watermelon (*Citrullus lanatus*) cultivar „Sugar Baby", the *Fusarium*-resistant cultivar „Charlestone" and with double-haploid pepper (*Capsicum annuum*) lines from breeding material. The latter ones are the susceptible DH-99-71 line and the DH-99-269, which contians the *gds* (general defense system) gene that ensures resistance against the *X. vesicatoria* bacterium. Plants used for experiments were grown in greenhouses under identical conditions.

For the infection of watermelon plants, we used conidium suspension of 10^7 conidium/ml concentration from the culture of *F. oxysporum f. sp. niveum* prepared in Czapek-Dox solution. For inoculation of pepper plants we applied a 48-h culture of *X. vesicatoria* bacterium to produce a suspension of 10^7 cell/ml concentration.

Roots of watermelon plants in the one-leaf development stage were cut under the soil on one side of the plant and sporule suspension of *Fusarium* was poured over them. Control plants were only inflicted with a wound. Bacterium inoculum was pressed into entirely developed leaves of pepper plants by a syringe on the abaxial leaf surface through the stomas without injury. Leaves of control plants were infiltrated with water.

Sampling

Based on our knowledge of the rapidity of the general defense reaction of plants, we started sampling immediately after inoculation and finished before the occurence of the symptoms. For the examination of watermelon, 15 plants (or certain parts of the plants) were used for each sample. For the examination of pepper, each sample was produced by homogenization of 10 leaf discs from infected parts of the tissue. On the other hand, paralell to samples collected from roots and sprouts of infected watermelon plants and leaves of pepper plants, we examined untreated control plant material also. Biochemical changes occuring in the course of pathogenesis were not compared with those in an uninfected plant in homeostasis, we would have rather compare the response of three different plant types to each other.

Chromatography

Fresh plant samples were homogenized by powdering them in liquid nitrogen. 0.25 g of each watermelon sample was weighed into Eppendorf tubes and 0.7 cm^3 0.02 per cent dimedon solution was added. In the case of pepper leaves, 0.6 cm^3 0.05 per cent dimedon solution was added to 0.6 g plant sample each. This suspension was centrifuged at 10 000 g for 15 min. Clear supernatants were suitable for Over-Pressured Layer Chromatographic (OPLC) separation. The HCHO content of watermelon samples was also determined by HPLC (High Performance Liquid Chromatography) analysis. Before HPLC analysis the supernatant was purified by filtering through a Samplex C-18 sample preparing patron following the above description of preparation.

OPLC detection of endogenous HCHO was carried out after the method of *Gersbeck* et al. (1989), while HPLC analysis was performed according to Sárdi & Tyihák (1994). The OPLC method of Gersbeck et al., besides analysis of dimedon adduct of HCHO, is also suitable for determination of the main natural generators of HCHO from the same sample on the same sorbent layer, by a subsequent, gradual

development by different systems of eluents. For qualitative and quantitative analyses, 20 µl of each watermelon sample and 25 µl of each pepper sample was applied on TLC and HPTLC silica gel 60 F_{245} chromatoplates (Merck) by a Hamilton syringe. In every case authentic substances (formaldemethone, TML, choline, carnitine, trigonelline, betaine), which are needed to qualitative and quantitative evaluation, were applied together with plant samples.

Results

In the course of our work on the watermelon – *Fusarium oxysporum f. sp. niveum* fungi and the pepper – *Xanthomonas vesicatoria* bacterium host-pathogen relationships, we tested reactions of plant species which do not contain specific resistance genes against biotic stresses. In our experiments we examined plant varieties and lines which have high level general defense system, due to restoring it during breeding work, and those which have ability for only low level defense, because of destructive effect of the kind of breeding which focuses on other intentions.

Watermelon – *Fusarium oxysporum f. sp. niveum* host-pathogen relationship

In the case of watermelon we studied the Sugar Baby cultivar, which is susceptible to the *Fusarium*, and the variety Charleston. Based on the method of testing, used in the course of *Fusarium*-resistance breeding, it is presumed that the resistance of Charleston is not ensured by specific resistance genes but by a well-restored general defense system.

Watermelon cultivars reacted to the *Fusarium* infection in different manners, accordingly they also proved different in respect to the biochemical processes taking place in them (*Figure 1*).

Before the inoculation, the quantity of choline, TML and HCHO was twice as much in the roots as in the shoots in both watermelon varieties. This shows that roots possess higher resistance potential and stronger protection. This is presumably because during sprouting of a seed the root emerges first, thus it is affected by strong biotic stress earlier, against which it must be protected. The shoot, which quickly emerges from the soil, is affected less by stress in this phenophase. The quantity of these compounds was identical in uninjured and injured control samples (data not shown) and those which were taken off at the moment of inoculation just after injuring the root. Both in the root and shoot of the *Fusarium*-resistant cultivar Charleston, we measured greater quantity of choline and TML than in the case of the less resistant Sugar Baby. This supports the relationship between the ability to endure stress and the quantity of quaternary ammonium compounds.

In both varieties, both the root and the sprout started to react to stress caused by inoculation at the same time, but the course of the reactions was different. The rhythm of change in the quantity of HCHO was similar to that of the two methylated compounds, as they are considered HCHO generators. Quantitatively, however, there was difference between the cultivars.

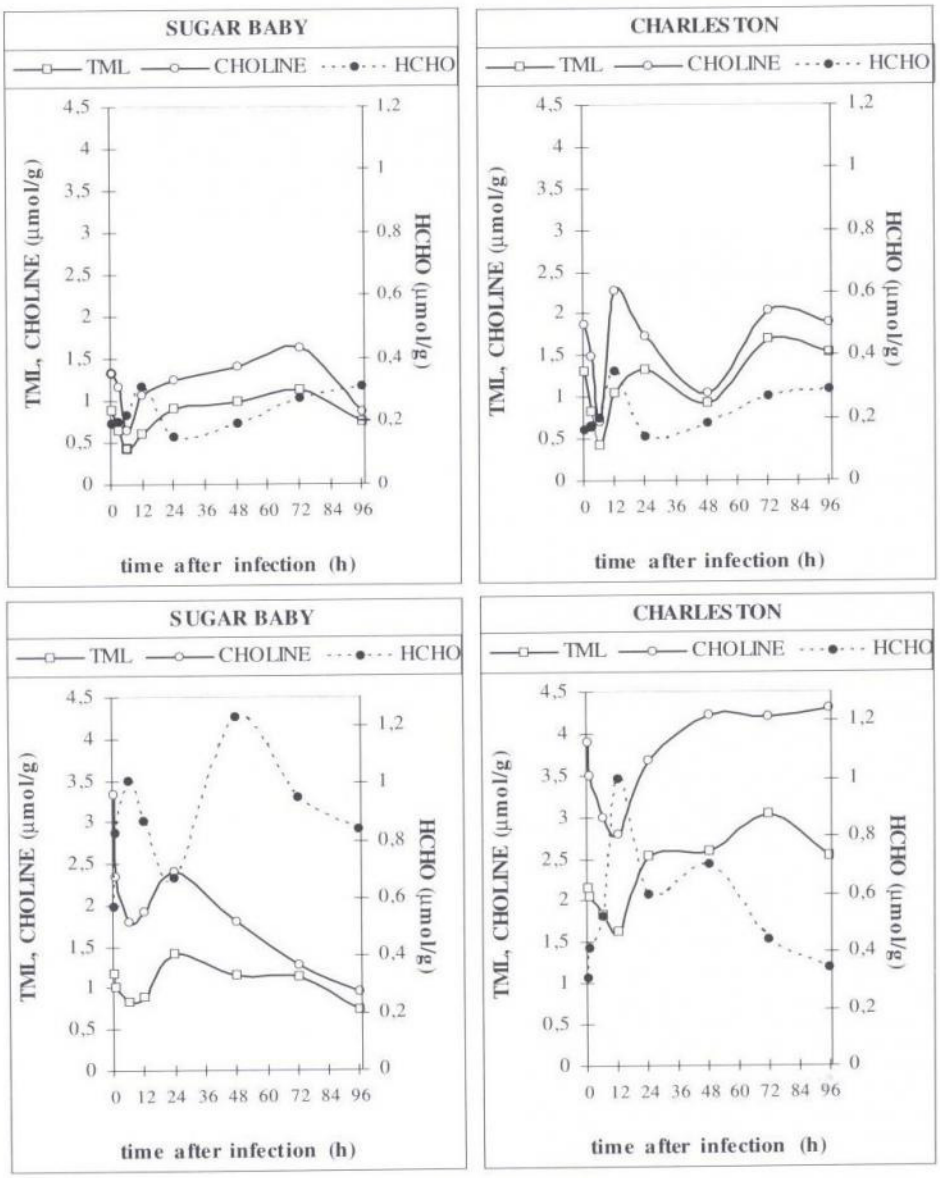

Figure 1 Time-dependent changes in the quantity of methylated compounds and formaldehyde (HCHO) in sprouts and roots of the susceptible Sugar Baby watermelon cultivar and the *Fusarium*-resistant Charlestone after artificial infection with the *F. oxysporum f. sp. niveum* fungus

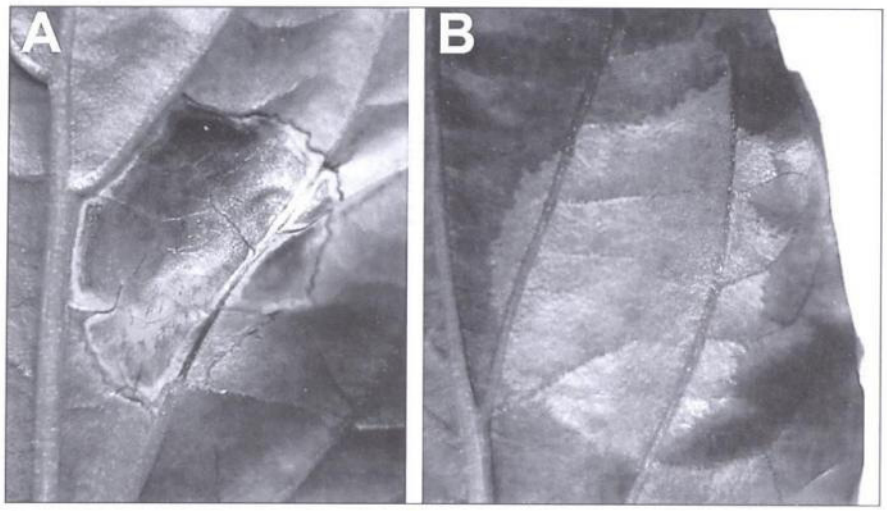

Figure 2 Tissue alterations of both of a leaf of a susceptible pepper line (A) and of a pepper line containing the *gds* gene (B), which ensures high level general defense reaction, as a consequence of infiltration with the *X. vesicatoria* bacterium

In roots of the susceptible Sugar Baby the quantity of HCHO reached its first maximum in the 6th h, at the same time as the minimum of choline and TML. In Charleston, these events occurred only in the 12th h, which can be explained by greater resistance potential.

In the shoots of both cultivars choline and TML also reached the lowest level in the 6th h, though by a smaller scale decrease, compared to the root. In Sugar Baby, a six-hour-long phase shift can be observed between the minimum value of methylated compounds and the maximum of HCHO in sprouts, which are far away from the starting-point of stress in the roots. In the case of the *Fusarium*-resistant Charleston variety, it is also true, but the volume of these quantitative changes is much larger.

In shoots of both cultivars, the highest HCHO concentration in the 12th h was followed by a quick fall to the initial level in the 24th h.

After a peak in the 6th h, HCHO content in the roots of Sugar Baby reached its lowest level in the 24th h – which is in the opposing period to the methylated compounds – then it jumped up to the second peak in the 48th h. In the 24th h the quantity of HCHO changed in opposite period to the concentration of methylated compounds in the inoculated roots of Charleston as well. It is an esential difference that, in contrast to the susceptible Sugar Baby, the second peak did not go beyond the first one, moreover it fell behind it.

We observed important differences between the changes in susceptible and *Fusarium*-resistant watermelon plants in the 48th h. In sprouts of the susceptible Sugar Baby, the quantity of choline and TML slowly reached the initial value in the 48th h, while in the roots it decreased continuously after making an attempt to restore the original conditions in the 24th h. The increase of the HCHO content in the root at same time refers to strong stress.

The *Fusarium*-resistant Charleston also showed striking changes in the 48th h. After the first minimum value in the quantity of choline and TML in the sprout, and following a fast increase, we experienced a continuous and significant fall which lasted from the 12th to the 48th h. At the same time we observed a strong increase in the roots. So, the quantities of choline and TML changed in the opposite direction in the two plant parts. In spite of this, the tendency in the quantity of HCHO is similar in the shoot and in the root: it is almost unchanged. It seems that it is almost independent of the changes of choline and TML. From this, it may be concluded that the quantities of choline and TML, synthetized in the plant parts above the ground, is transported to the roots. If we accept that the increased quantity of methylated compounds in the roots originates from transport, we may get explanation of the independent behaviour of the quantity of HCHO. In the 48th h the level of choline and TML in the root exceeds the initial value, as a consequence of the transport coming from the shoots, and this level remains unchanged until the end of the duration of experiment. It is notable that at the same time, the quantity of HCHO shows a strong decrease. From the 48th h the quantity of choline and TML begins to be restored in the shoots as well, which ensues already in the 72th h. During the saturation,the level of HCHO shows a slight increase.

In the shoot of Sugar Baby – after a continuous but quite slow increase having started in the 6th h – choline and TML began to fall dramatically in the 72nd h. In the 72nd h, a decrease in the quantity of the two methylated compounds was observed in the shoot of the Charleston too, but it was much slower than in the case of the Sugar Baby.

Pepper – *Xanthomonas vesicatoria* host-pathogen relationship

With respect to the sensitivity of the studied biochemical processes, we performed our experiments on genetically equalized double-haploid pepper lines, which are the DH-99-71, a pepper line susceptible to the *X. vesicatoria* bacterium (*Figure 2a*) and the DH-99-269, which contains the *gds* (general defense system) gene. Due to the *gds* gene, the DH-99-269 line is able to give such a high level general defense reaction that it has complete protection against the *X. vesiactoria* bacterium even without specific resistance genes (*Bs-1, Bs-2, Bs-3*). While specific resistance genes ward off attack of pathogens by a reaction which causes cell and tissue destruction, the *gds* gene ensures tissue packing which is based on preserving cells (*Figure 2b*).

Since we intended to examine the relationships between transmethylation and biotic stresses exclusively, we ruled out the possibility of abiotic stresses, which may be caused by the method of inoculation. We pressed the bacterium inoculum into the intercellular space through the stomas on the abaxial surface of the leaf without injuring the tissues.

In the case of the susceptible DH-99-71 pepper line, there was no significant difference between the water-infiltrated

tissue used as control, and the leaf sample which was taken off immediately after the infiltration with bacterium suspension (*Figure 3a*). In the first h of the inoculation choline content elevated, then it showed a fairly significant continuous decrease until the 12th h. The HCHO level continuously decreased until the 6th h then it rose suddenly, causing the two curves to intersect each other before the termination of the 12th h.

In the DH-99-269 line, which contains the *gds* gene and possesses high level general defense reaction, the level of choline in tissue samples taken off just after the infiltration was much lower than in the water-infiltrated tissues (*Figure 3b*). The rate of decrease continued until the end of the first h, after which it did not change significantly. But it increased clearly from the 6th to the 12th h. The quantity of HCHO did not show an important difference between water-infiltrated control tissues and tissue samples collected just after the infiltration with bacterium. It did, however, rise sharply and it reached a maximum within an hour. The curves showing the quantities of choline and HCHO intersect each other in the 30th min. The quantity of HCHO showed a continuous decrease from the 1st to the 12th h in such a way that the second point of intersection of curves occured in the 6th h.

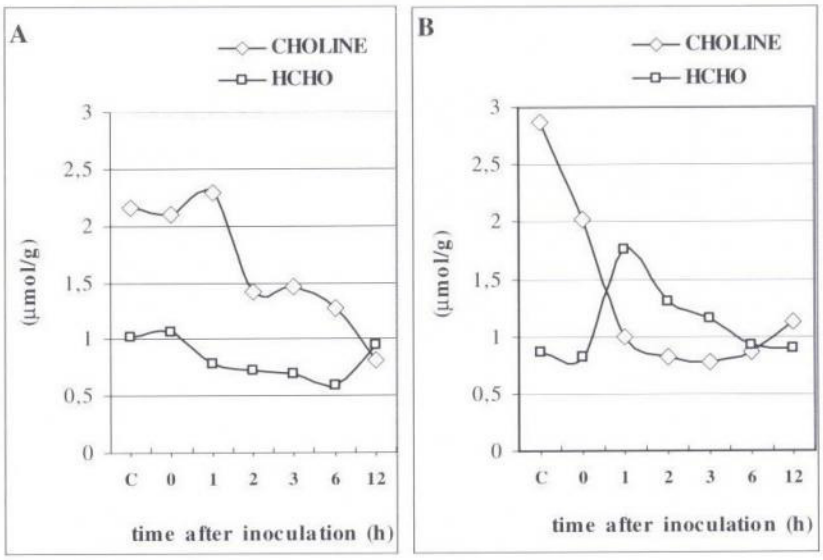

Figure 3 Changes in the level of choline and formaldehyde (HCHO) in leaves of a susceptible pepper line (A) and of a pepper line containing the *gds* gene (B) as a consequence of artificial infection with the *X. vesicatoria* bacterium
(C = water-infiltrated control)

Discussion

We have examined the relationship between transmethylation processes and the general defense reaction of plants. This has increased our knowledge of both the processes regulated by transmethylation and the general defense reaction of plants.

Watermelon

Through studying changes in the quantity of choline and TML, and in connection with them quantity of HCHO in the roots and sprouts of the susceptible Sugar Baby and the resistant Charleston after artificial infection with *Fusarium*, we produced the following conclusions. Regardless of resistance against the *Fusarium*, the biochemical changes of different extent, which took place up until the 6th h at the same time in the root and sprout of both cultivars, are not consequences of *Fusarium* infection, as this is too short an interval following the inoculation. Rather these are consequences of cutting the root to promote the *Fusarium* to penetrate, which is an abiotic stress. Besides, stress tolerance of root of the *Fusarium*-resistant Charleston in notable.

Changes which took place in the 48th h, regardless of the resistance of the varieties, are unambiguously a consequence of germinating conidia of *Fusarium* and the biotic stress which evolved during the course of infection. In susceptible plants this is not perceptible on the basis of quantity of choline and TML, but the rise of HCHO in roots (which presumably originates from other methylated compounds), demonstrates the start of the process of pathogenesis excellently. In sprouts of plants – whose roots are resistant and which resistance needs a complex genetic background – quantity of choline and TML increased considerably as a result of stress. At the same time a transport started towards those parts that were directly exposed to stress, namely the roots. This is not typical of susceptible cultivar however.

Pepper

We examined quantitative changes of choline, and in connection with it the endogenous HCHO, in a pepper line susceptible to the *X. vesicatoria* bacterium and another which contains the *gds* gene and has a high level, restored general defense system which has not been known in resistance breeding by this time and was discovered by Hungarian researchers (*Szarka & Csilléry*, 1995).

In the case of the susceptible DH-99-71 pepper line, neither the water-infiltrated control sample nor the bacterium-infiltrated, immediately picked leaf showed significant changes in the quantity of choline and HCHO. In our opinion the increase in the quantity of choline in one h after inoculation can be first explained by the transport coming from the neighbouring tissues that have not been infiltrated (*Szarka* et al., 2002). Following this we could observe a continuous decrease in the quantity of choline without any increase in the HCHO level. The important role of HCHO in the defense against pathogens is shown by the fact, that for lack of it, the pathogenesis begins.

Contrary to this, in the DH-99-269 line, which contains the *gds* gene, we experienced a large difference in the

quantity of choline between the tissue sample of the water-infiltrated control and the leaf removed just after inoculation. The role of the general defense system in plants is similar to that of human and animal immune systems. Its exceedingly high reaction speed, owing to its physiological function, manifested itself one h after inoculation in the fast de-methylation of choline. As a consequence of demethylation, which started at the moment of inoculation, the quantity of HCHO began to rise immediately and reached its maximum value in one hour. With this, the defense reaction was completed, the quantity of HCHO started to fall and at the same time a slow increase in the level of choline could be observed.

As a result of our investigations we state that transmethylation processes are suitable to characterize abiotic and biotic stresses, which affect plants, and to follow plant reactions. The inoculation methods in resistance breeding which cause injury, strongly inhibit the operation of the defense system of plants and, as a consequence of this, they can produce incorrect results. A strong abiotic stress caused by an injury runs through on the whole of a juvenile plant. But if we use a kind of inoculation which imitates natural infection, pathogens cause minor stress of only local character, particularly at the beginning of an infection.

Resistance genes encoding either the general or specific defense reactions strongly reduce exposure to biotic stresses by ensuring fast reactions. In their absence, the sensation of stress caused by pathogens develops only later, after the propagation of pathogens in the plant.

Changes in the levels of choline, TML and HCHO are synchronized at the immediate site of the stress effect. In more distant parts of the plant a phase shift of the quantitative changes in HCHO can be observed. The full complex of transmethylation processes can be characterized well through changes in the HCHO content. The ability of plants for the general defense tightly correlates with the quantity of choline and TML. Their levels determine the stress tolerance of cultivars.

The plants which have high level, restored general defense system are fairly suitable for studying the patho-physiological role of transmethylation processes intensely. Therefore, we consider it necessary to extend our examination of the quantitative changes of additional methylated compounds. Studying transmethylation processes seems suitable to prove functional divergences between the fundamental general defense reaction of plants and the specific resistance genes.

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