Plant immunity (synthesis and interaction of sciences): ROI, mutagen action, genetical instability, and plant immune responses

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Barley *tweaky spike* (*tw*) mutants are useful for plant immunity investigations, especially of the processes that take place in or around the germinating grain/seed, because of three peculiarities of germinating *tw* grains: (i) high frequency of moldy grains and analysis of micromycetes on/in the grain, (ii) chromosome instability (higher frequency of chromosome aberrations and SCE), and (iii) stronger EPR-signal in the seed-coats of *tw* mutants in comparison to the normal type. The problem is the same for both plants and mammalia: mild chromosome instability, immunodeficiency are characteristics of human diseases with a disturbed ROI (reactive oxygen intermediates) balance.

For the immunodefiency of *tw* mutants, important is also a specific structure of the ear. The mutants are strongly sensitive to the pathogens *Claviceps purpurea*, *Ustilago nuda* for which the structure of the ear/flower is the dicisive factor. It has been determined that *tw* mutants are homeotic: lodicules are converted to pistils or stamens.

Key words: plant immunity, germinating grains, moldy grains, EPR-signal, chromosome instability, homeotic barley mutants

INTRODUCTION

Immunodefiency is one of the main peculiarities of the barley *tweaky spike* (*tw*) type mutants. It was displayed in many-year investigations with different test-systems to various pathogens. First, an exceptional sensitivity to micromycetes was noted in field observations as a high frequency of plants affected by *Claviceps purpurea* and *Ustilago nuda* [1, 2]. The second phenomenon employed as a proof of an exceptional sensitivity of *tw* mutants is the high frequency of moldy germinating grains of *tw* barley [2, 3]. That peculiarity was discovered in cytogenetic trials: it was impossible to grow seedlings for root tips fixation without grain sterilization of the *tw* mutants.

Recently L. Balčiūnienė [4] has tested the sensitivity of nine allelic *tw* mutants to three different pathogens: *Helmintosporium tere's*, *Puccinia simplex*, *Erysiphe graminis*. Only one mutant was found nearly as resistant as the normal type to *H. tere's*, two – to *E. graminis*. Others were more susceptible to the pathogens tested.

An impetus to return to the problem of the immunodeficiency of *tw* type mutants was given by recent findings in plant immunology and immunogenetics concerning the role of the reactive oxygen intermediates (ROI) such as H_2O_2, $OH^{\bullet}$, $O_2^{\bullet -}$ and others, in plant immune responses and acquired resistance to the pathogen [5–10]. These findings were in agreement with our data about EPR-analysis of barley grains. Increased EPR signal was observed in the grains of *tw* mutants. It was especially high in the seed-coats [11, 12]. However, these investigations were performed in connection with genetical instability of several barley *tw* mutants (*tw*, tw_1, tw_2). A significant level of reversions is also among the main peculiarities of these *tw* type mutants [1].

An especially intriguing fact is that ROI (H_2O_2) [13] and plant defence peptides of low molecular weight (5kDa) – defensins [14] are secreted outside the germinating seeds, making, protective zone around them.

The EPR signal was stronger in the seed-coats of the grains of *tw* mutants [12], and, on the other hand, the genetical instability of those mutants was also expressed by the higher level of chromosome aberrations and SCE (sister chromatid exchanges) in the meristematic cells of root tips in germinating grains [1, 11, 15, 16].

We noted that a specific complex of traits is a common characteristic of human diseases accompanied by a disturbed balance of the free-radical level. To such traits belong mild chromosome instability, decreased DNA repair, immunodeficiency, sensitivity to mutagens [1].

The role of ROI in immune responses may explain all these observations and is the same in plants and in animals.

MATERIALS AND METHODS

The first *tw* mutant was induced by the author about 30 years ago after treatment with ethyleneimine and detergents in the initial cv. 'Auksiniai II'. The other two allelic mutants were also isolated from cv. 'Auksiniai II'. So, they may be evaluated as isogenic lines. Several new allelic *tw* type mutants were also induced in cv. 'Auksiniai 3'. From M_3–M_4, mutants *tw*, tw_1 and tw_2 were divided into sublines – the progenies of separate mutant plants.

Mutants *tw*, tw_1, tw_2 are genetically unstable, and revertants to the normal type $tw \rightarrow Tw$ arise. Each revertant was compared with the subline from which it had been derived.

During all years of studies the mutants, revertants and initial barley cultivars were planted under the same conditions without pesticides.

The mode of inheritance (allelism, interaction in complementation test, genetical recombination) was determined in hybridization trials between *tw* mutants, normal type and markers for other genes. The nature of revertants was also examined by hybridization with the initial mutant subline.

Sensitivity to *Claviceps purpurea* Tul. and *Ustilago nuda* (Jens.) Rostr. was determined in many-year field observations. Artificial inoculation with *Ustilago* spores was carried out in a two-year experiment. The ears were specially prepared: the ear-covering leaf and the awns were removed. Inoculation was made in a special glass reservoir in vacuum or in a dry mode on a specially created provocative background.

The frequency of moldy germinating grains was determined in Petri dishes in a thermostat at 24 °C in the dark. In each Petri dish 10 grains were placed. It is a very important condition, because in a more dense culture grains infect from one another.

Micromycetes composition was analysed three years in turn. For their external determination, grains were germinated without sterilization. For determination of internal micromycetes grains were sterilized for 1 min with 96% ethanol. Then the grains were washed with sterilized distilled water. All materials and dishes were used sterile.

Evaluation of spontaneous **chromosome aberrations** in the meristematic cells of the root tips was made in temporary preparations stained with acetoorseine. Material was fixed in Carnoy solution (ethanol : acetic acid 3:1), and a differential staining of chromosomes and **sister chromatid exchanges (SCE)** were carried out by N. V. Luginin's method [17]. This method allowed a simultaneously scoring of C-bands and SCE. The distribution of SCE among barley chromosomes was noted.

Free radicals (FR) testing was carried out at the Institute of Chemical Physics of the Russian Academy of Sciences on a 3 cm RE-1306 radiospectrometer. The amplitude of EPR signal was measured at room temperature. The content of FR was expressed in relative units to standard. Differences in the weight of samples were not greater than 5%. Analysis was made in desiccated material. Desiccation was performed in two different ways: (1) drying at 105 °C for absolute weight; or (2) lyophilisation in a lyophilizator. In both cases the dried material was stored in a desiccator over CaO.

Superoxid dismutase (SOD) isozymes were analysed by disc electrophoresis in polyacrilamide gel according to Davis and Ornstein [18].

Isolation of DNA followed a modified protocol of Dellaporta et al. [19]. RAPD and RAMP analysis [20, 24] with random primers and microsatellite primers was performed, and the results were analysed with the TREECON program [22].

Development of the ear/flower structure was analysed as early as possible from the fifth leaf stage to full development of the flowers. Observations were made on a SMZ-143 stereo zoom microscope (Motic ®).

RESULTS AND DISCUSSION

1. Genetical instability and immunodefiency are related problems

The maintenance and investigation of many induced mutants depended on the most 'fashionable' problem of a certain period of time. Barley *tw* mutants are not an exception from that rule.

The first mutant *tw* was induced in the period [23] when the problem of genetical instability and search of mutator genes, mutants which are sensitive to mutagens, was at the top.

The pretext for investigation of barley *tw* mutants and their genetical analysis, determination of the chromosome aberrations and SCE frequency, free radical (FR) content on an EPR spectrometer was not the immunodeficiency of those mutants, but their genetical instability. In the progenies of three allelic mutants, *tw*, tw_1 and tw_2, normal plants arise perma-

nently. Investigations were aimed at showing that these plants are *tw*→*Tw* revertants and at explaining the genetical instability of those mutants. After establishing fact that mutations in the *tw* locus are pleiotropic, an interest to revertants arose, and the second task of the study was solved. It was proposed that revertants may not mean an exact return to the initial normal type, several characters from the pleiotropic trait complex may be preserved. A higher protein content in the grains of *tw* mutants was determined, and revertants with restored productivity and higher protein content were obtained [1, 24].

The other manifestation of genetical instability was a slight but statistically significant increase in chromosome aberrations and SCE frequency [11, 12, 15, 16]. Even after nearly 30 years since *tw* induction it still has an increased frequency of chromosome aberrations [25].

Distribution of SCE among barley chromosomes was analysed, and a higher SCE frequency in meristematic root tip cells of barley *tw* mutant may be attributed to chromosome 6 and in several cases to its q arm [16].

One of the supposed causes of the both manifestations of genetical instability may be a higher content of free radicals. Such an explanation may be especially real for chromosome instability. At that time it was already known that human diseases with disturbed FR balance were characterizied also by a mild chromosome instability and decreased DNA repair [see as review, 1]. That supposition was confirmed by experimental data: the increased levels both of chromosome aberrations and FR content were characteristics of *tw* type mutants.

However, analysis of the distribution of FR within the grain gave surprising results. A higher FR level was found not in the grain embryos as expected, but in the seed-coats [12]. So, it was supposed that problem reached a deadlock for FR as the main cause of the higher level of chromosome aberrations and frequency of moldy germinating grains in *tw* mutants. However, the fact that FR are concentrated in the seed-coats of *tw* mutants is the main reason for revising the data on the immunodeficiency of barley *tw* mutants to fungal pathogens.

2. **Immunodeficiency of *tw* mutants and plant response to pathogen and stress conditions**

Interaction between mutagenesis and plant immune responses can be more intimate and unexpected. Recently the results of many-year investigations in the laboratory of L. A. Hadwiger et al. [26] have been summarized, and a conclusion was made that the promoters of *PR*-genes may be activated with DNA-damaging agents such as H_2O_2, UV, mitomycin C, actinomycin D, etoposide, netropsin and others.

PR (*pathogen-related*) genes are activated by pathogen and cause induction of resistance, called SAR (systemic acquired resistance) [7, 27, 28]. However, the same *PR*-genes were induced in *Pisum sativum* by the pathogen *Fusarium solani f.* sp. *phaseoli* and also with DNA-damaging agents [26]. It was found that the list of the factors capable of inducing *PR*-genes is long. These genes are also activated by plant wounding, aphids and other herbivorous insects, as well as by different forms of stress [29, 30].

The finding of the L. A. Hadwiger group [26] is the clue to the question why chromosome instability, immunodeficiency and inhibition of DNA repair are also characteristics of the human diseases with disturbed ROI balance, including chronic inflammatory diseases caused by pathogens [1].

Mutagenic ROI products may activate equally *PR*-genes and genes that determine DNA repair.

Although the action of the pathogen on DNA repair in plant has not yet been demonstrated, several homologs to human excision repair genes have been reported, the *MSH–2* gene homolog of mismatch repair among them [31]. Mutations in that gene are mutators and cause genetical instability.

The other recent finding related to barley *tw* mutants is the fact that roots of intact plants and germinating seeds are capable of releasing H_2O_2, defensins into the surrounding medium, even in the absence of pathogen attack or other stress elicitors [13, 14]. So, the increased level of the EPR signal in the seed-coats may be a defense response to grain-surrounding micromycetes.

In collaboration with Dr. R. Mačkinaitė from the Institute of Botany (in Vilnius), a detail analysis of about 70 exogenous and endogenous micromycetes was done, with the hope to find the micromycetes that attack specifically *tw* mutants. Under the influence of the recent findings discussed above, the results of those investigations were summarized anew (Table). It became obvious that only the frequency of one fungus, *Alternaria alternata*, increased in the grains of the *tw* mutants in all years of.

Attempts to decrease the frequency of moldy germinating grains with antioxidants (ascorbic acid, gluthatione and cysteine) was ineffective. Only several concentrations of cysteine reduced the frequency of moldy grains [32].

H_2O_2, superoxides and the other ROI are at the same time DNA-damaging and PR-inducing factors [26]. However, their role in pathogen defence responses is multifarious, and ROI act in the earliest phase of the plant-pathogen interactions [9, 10, 13]. The early phase of pathogen attack can induce a rapid production of ROI by plant cells, the so-cal-

Table. **The most frequent internal micromycetes in grains of barley mutants *tw* type and their revertants (R) in comparison with the normal type of barley cv. 'Auksiniai II'**

Micromycetes	AII	*tw*	tw_1	tw_2	*tw*–R	tw_1–R	tw_2–R
			1992				
Total number	15	18	11	11	19	9	11
Chaetomium sp.	0	9.4 ± 1.3	2.0 ± 1.4	0	11.2 ± 1.4	3.0 ± 1.7	5.0 ± 2.2
Mycelia sterilia	0	3.6 ± 0.8	17.0 ± 3.7	4.0 ± 1.8	4.4 ± 0.9	6.0 ± 2.4	8.0 ± 2.2
Paecilomyces sp.	5.0 ± 2.2	15.6 ± 1.6	12.0 ± 3.2	10.0 ± 3.0	3.0 ± 0.8	12.0 ± 3.2	4.0 ± 1.9
Alternaria alternata	18.0 ± 3.8	32.6 ± 2.1	43.0 ± 4.9	39.0 ± 4.9	25.6 ± 1.9	38.0 ± 4.8	60.0 ± 4.9
Fusarium: number of species	2	5	3	4	6	3	2
total frequency	15.0 ± 2.4	14.2 ± 0.5	20.2 ± 2.3	17.0 ± 1.9	19.2 ± 0.7	18.0 ± 2.2	21.0 ± 2.4
			1993				
Total number	10	16	15	14	18	15	15
Mucor sp.	1.0 ± 0.9	1.8 ± 0.6	4.0 ± 1.4	10.0 ± 3.2	0.8 ± 0.4	5.5 ± 1.6	4.0 ± 2.0
Trichothecium roseum	1.0 ± 0.9	17.4 ± 1.7	9.5 ± 2.1	23.0 ± 4.2	19.0 ± 1.8	0	25.0 ± 4.3
Alternaria alternata	12.0 ± 3.2	22.4 ± 1.9	25.0 ± 3.1	23.0 ± 4.2	31.4 ± 2.1	15.0 ± 2.5	25.0 ± 4.3
Drechslera graminea	0	3.4 ± 0.8	4.0 ± 1.4	1.0 ± 0.9	3.6 ± 0.8	1.4 ± 0.7	3.0 ± 1.7
Stemphyllium botryosum	0	2.4 ± 0.7	5.0 ± 1.5	3.0 ± 1.7	6.0 ± 1.1	0	6.0 ± 2.4
Cylindrocarpon didymum	0	0.8 ± 0.4	4.5 ± 1.5	0	2.6 ± 0.7	0	1.0 ± 0.9
Fusarium: number of species	5	8	9	6	11	7	6
total frequency	42.0 ± 2.2	34.0 ± 0.8	25.5 ± 1.0	29.0 ± 2.0	36.0 ± 0.5	24.1 ± 0.8	34.0 ± 1.6

led 'oxidative burst' that is closely resembling the 'oxidative burst' in pathogen-activated mammalian phagocytes. Homology to mammalia is also observed in the mechanism of that early ROI production. In activated mammalian phagocytes, ROI are produced by the inducible NADPH oxidase redox system in the plasma membrane. Two NADPH oxidase components, $gp91^{phox}$ and Rac, are cloned in the plants. The latter induces HR (hypersensitive response) and apoptosis (programmed cell death) necrosis. So, necrosis restricts the spreading of the pathogen in the plant tissues [8].

The homologeneity of the mammalian and plant 'oxidative burst' is so great that the same drugs may operate against the pathogen in the mammalia and in the plants. Salicylic acid (SA) and its derivatives were effectively used for increasing the resistance to plant pathogens [33].

Induction of SA synthesis is one of the important plant responses to a pathogen. SA action in the plants is double: it acts as a regulator of the *R* and *PR*-genes and at the same time is a signalling molecule in SAR (systemic acquired resistance). SAR represents resistance of the whole plant, in distinct sites remote from a pathogen-infected tissue. So, the signal transport from a pathogen attack site to the other tissues is necessary. SA is one of the signalling molecules [34].

The early production of ROI causes also destruction of the cell membrane, induction of the other groups of the proteins, products of the *R*-genes. The R-proteins determine resistance to pathogen by the interaction „gene-for-gene" mode: *avr* (avirulence) product of the pathogen interacts with the R-protein of the plant. By some properties R-proteins resemble mammalian immunoglobulins. Both protein groups have hypervariable regions, by which they are capable of a fast-adapting recognition of *avr* signals or antigens, respectively [8, 34]. R-proteins and immunoglobulins differ in their structure. So, there is a phenomenon, common for both plants and animals, which E. M. Meyerowitz called 'evolutionary logic' [35].

ROI act also as signalling factors in the late phases of defence responses [8, 9, 34].

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AUGALŲ IMUNITETAS (MOKSLŲ SINTEZĖ IR SĄVEIKA): RDT, MUTAGENŲ VEIKIMAS, GENETINIS NESTABILUMAS IR AUGALŲ IMUNINIS ATSAKAS

S a n t r a u k a

Miežių mutantų *tweaky spike* savybės svarstomos po naujų atradimų augalų imunogenetikoje. Ypatinga RDT (reaktyviųjų deguonies tarpininkų) svarba.

Miežių *tw* mutantai derina tris svarbias imuniteto tyrimams savybes: yra jautrūs mikromicetams; jų grūduose didesnis EPR-signalas, tad aktyesni RDT; mutantams būdingas genetinis nestabilumas, (ypač svarbus chromosomų nestabilumas, pasireiškiantis didesniu chromosomų aberacijų ir seserinių chromatidžių mainų dažniu).

Dygstantys mutantų grūdai yra puikus modelis augalų imunitetui tirti, ypač panaudojant tyrimams dygstančių grūdų pelėjimą. Apžvelgiamos augalų imuniteto problemos, susijusios su laisvųjų radikalų susidarymu ir mutagenų poveikiu. Palyginami gyvūnai ir augalai.