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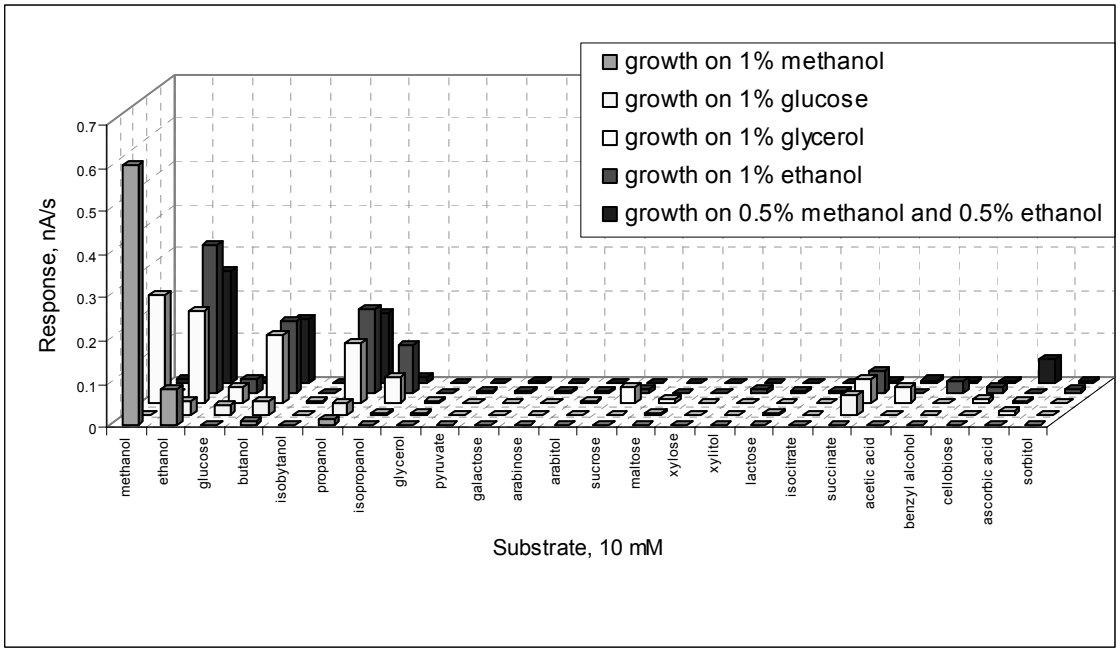

Figure 1: Substrate specificity of the biosensor based on *P. angusta* VKM Y-2518 under the cultivation on various substrates

The growth on glycerol leads to some decreasing of the signals to methanol and increasing of the response to ethanol; in fact, the values of the responses to ethanol and methanol become comparable. This variant of the cells is also characterized by the broadening of the specificity similar to one observed under cultivation on ethanol. It's probably that the glycerol acts as the inductor or repressor neither for AO nor for ADH, that leads to expression of the both enzymes, although in less amounts (by the value of response to methanol) than under the cultivation on the specific substrates.

The bioreceptor fabricated from the cells grown on glucose was found to be non-efficient from the viewpoint of the sensor functioning. The sensor based on the such cells was characterized by low responses to all tested substrates. It is indicative of low level of the expression of the both alcohol-catabolizing enzymes in the presence of glucose.

4. Conclusions

1. The substrate specificity of sensors based on cells of methylotrophic yeast *Pichia angusta* VKM Y-2518 under cultivation on various substrates has been investigated.
2. It was found that the highest selectivity is characteristic for the sensor based on cells grown on methanol; this sensor demonstrates high value of response to methanol and could be applied for the methanol detection.
3. The sensors incorporating the bioreceptors based on cells cultivated on ethanol, glycerol and ethanol-methanol mixture are characterized by broad range of detectable substrates. These sensors could be used, e.g., for determination of biological oxygen demand.
4. The sensor fabricated from the cells grown on glucose demonstrated low responses for all tested substrates and thus considered as unpromising.

References

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BLACK MEDIC AS A MODEL PLANT TO STUDY OF ARBUSCULAR MYCORRHIZA EFFICIENCY

Yurkov A.P.[1], Jacobi L.M.[1], Kojemyakov A.P.[1], Semenov D.G.[2]

[1]*All-Russian Research Institute for Agricultural Microbiology, 189620 St. Petersburg-Pushkin-8, Podbelsky Sh. 3, Russia*

[2]*Russian State Hydrometeorological University, 195196 St. Petersburg, Malookchtinsky prospect 98, Russia*

yurkovandrey@yandex.ru

Keywords: arbuscular mycorrhiza, symbiotic efficiency, black medic, *Glomus intraradices*, polymorphism

1. Introduction

Arbuscular mycorrhiza (AM) is a widespread plant-microbe association. The mycorrhiza plays a key role in plant health and productivity by increasing mineral (especially phosphate) nutrition, and optimizing of plant hormone potential, promoting resistance of plants to root pathogens and abiotic stresses. The present study is aimed to analyze the role of the host plant in the efficiency of AM in the aspect of adaptation to low phosphate (P) level in soil. So plant polymorphism estimation, analysis of correlations and selection of plant populations are the main topics of the presented part of our research. The results can be used to select the plant genotypes with a high symbiotic potential.

2. Materials and Methods

The subject of the study is black medic (*Medicago lupulina*) - self-pollinating diploid species characterized by broad ecological amplitude. Black medic is used as a grazing crop, planting filler, and green fertilizer. This species shows a high seed production. Two wild-growing populations (p. Pavlovskaya and p. Yuntolovskaya from Leningradslaya Region) and two cultivar-populations (cv. Mira from Moscovskaya Region and cv. ARFI32 from Kazakhstan) were analyzed in aseptic pot experiment under conditions of low P content in sterilized soil. The highly efficient strain of AM-fungus *Glomus intraradices* was taken to inoculate the plants. All experimental conditions were controlled to prevent the inoculation with rhizobia. The characteristics of plant productivity (dry matter, shooting,

plant height) and mycorrhization (frequency of mycorrhiza, intensity of mycorrhiza, arbuscule and vesicle abundances in roots and in mycorrhiza) as well as leaf surface area and P content per plant were recorded at 88th day after sowing. The mycorrhization was examined in the roots stained by trypan blue (J.M. Phillips, D.S. Hayman, 1970). The preparations were studied under light microscopy (A. Trouvelot et al., 1986). Both quantitative and statistic estimation of the mycorrhization were improved by the original computer program (A.P. Yurkov, unpublished data). Coefficients of variation (*Cv*), asymmetry (As), and excess (Ex) for characteristics of plant productivity and mycorrhization were estimated according to P. Hedrick (2003).

3. Results and Discussion

Interpopulation variation: Differences between analyzed populations in symbiotic efficiency were significant (see Fig. 1). On the Fig. 1: "–AM" – nonmycorrhizal plants in control sample; "+AM" – mycorrhizal plants in experimental sample. The obligate mycotrophic form (ARFI32), characterized by dwarf symptom in uninoculated control under low P level in soil was selected (see Fig. 1).

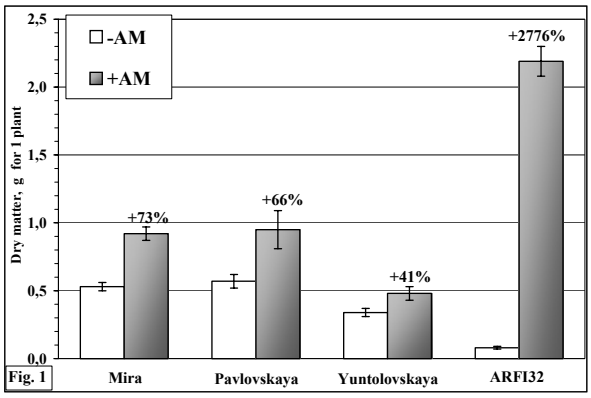

Figure 1: Differences of dry matter in black medic populations at 88th day after sowing.

Intrapopulation variation: Significant polymorphism for productivity in distinct populations was observed (see Table 1), as well as polymorphism for characteristics of mycorrhization. As a result p. Pavlovskaya had a highest variability in following characteristics: 1) dry matter of mycorrhizal plants (*Cv* = 89%, Table 1); 2) frequency of mycorrhiza (from 0 to 96%).

Table 1: Intrapopulation variation of black medic.

Statistical	Plant hight		Shooting		Dry matter	
parameters	–AM	+AM	–AM	+AM	–AM	+AM
	Caltivar-population Mira					
C_v	29[1]	61	38	21	39	39
As	2,6	1,5	-0,8	-0,3	0,0	0,9
Ex	10,3	4,4	-0,2	-0,3	-0,5	0,2
	Wild-growing population Pavlovskaya					
C_v	43	78	61	39	53	89
As	1,3	1,7	0,5	-0,3	0,7	1,6
Ex	1,6	2,0	-0,8	-0,7	-0,3	1,4
	Wild-growing population Yuntolovskaya					
C_v	39	65	53	39	58	57
As	0,4	1,4	0,4	0,2	0,4	1,4
Ex	-1,0	1,7	-1,1	-0,7	-0,9	1,8
	Caltivar-population ARFI32					
C_v	32	10	47	12	60	29
As	0,5	0,0	1,6	-0,8	1,8	0,0
Ex	0,6	-0,6	2,5	0,9	5,7	-0,6

The observed changes in the structure of distinct populations upon inoculation suggest genetic polymorphism for the efficiency of symbiosis with AM-fungus (see Table 1). In Table 1: the experimental values into grey cells have significant ($P<0.05$) differences in comparison to uninoculated control, indicated intrapopulation genetic polymorphism of black medic for AM-efficiency.

Correlations: The correlation analysis was carried out to examine interrelations between productivity and mycorrhization. As a result of analysis at 88th day after sowing in p. Pavlovskaya the coefficients of correlation between characteristics of productivity and mycorrhization were positive and significant ($P<0.05$), including correlations between dry plant matter and arbuscule abundance. According to analysis of plants in p. Pavlovskaya the significant correlations between P content in the plant and characteristics of mycorrhization were not found ($P>0.05$). On the other hand in p. Yuntolovskaya, cv. Mira, and cv. ARFI32 the linear correlations between productivity and mycorrhization were not shown up, but they had significant ($P<0.05$) curvilinear links. The arbuscule abundance was shown to depend on dry plant matter in these populations, but not otherwise.

Selection of plant populations: Wild-growing population Pavlovskaya was selected to isolate contrasty plant genotypes differed in symbiotic activity with *Gl. intraradices* and so in order to produce lines with high AM-efficiency. With this design the analysis of progeny of p. Pavlovskaya is planed.

Cultivar-population ARFI32 – obligate mycotrophic symbiont – was selected as a model plant to study the mechanisms, regulating AM-efficiency under conditions of low P content in soil. The pilot chemical mutagenesis of ARFI32 plants was done in order to isolate symbiosis-defective plant mutants. Currently the phenotypic analysis for productivity and mycorrhization of M2 and M3 progeny is in process. The research of dynamics of productivity, mycorrhization and photosynthesis is begun in this population under conditions of two P levels in soil (with fertilization and without fertilization). Noted, that host-plant response to inoculation with *Gl. intraradices* occurred already on early phases of plant ontogenesis.

4. Conclusions

The results of the present study will forward basic research, connected with examination of the plant mechanisms regulating the efficiency of beneficial symbiotic plant-microbe interactions in arbuscular mycorrhiza.

The data on black medic variability profit for selection of lines with a high AM-efficiency in order to create the high-productive symbiotic systems: “plants – microorganisms”.

The practical application of cultivars with a high symbiotic potential would allow to reduce the rates of mineral fertilization (especially phosphate) and would help forward the development of sustainable agriculture.

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PROTEIN BASED FRET ASSAY FOR DETECTION OF ANTHRAX LETHAL FACTOR PROTEASE ACTIVITY

Zakharova M.Yu.

M.M. Shemyakin & Yu.A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences Ul. Miklukho-Maklaya, 16/10, 117997 GSP, Moscow V-437, Russia

Keywords: anthrax, lethal factor, fluorescent proteins, FRET

Abstract

Lethal factor (LF) is Zn+-dependent proteolytic component of the tripartite anthrax toxin that is responsible for cleavage of host cellular proteins and for fatal outcome of anthrax. In order to prevent LF-mediated intoxication, it is critical to develop inhibitors of LF proteolytic activity. Peptide LF substrates developed as analytes for high-throughput screening (HTS) of LF inhibitors are expensive and do not permit monitoring of LF activity in mammalian cells. We developed simple and inexpensive assay for rapid assaying of LF activity, using the protein-based fluorescence resonance energy transfer (FRET). We employed a genetically engineered cassette containing cyan and yellow fluorescent proteins (FP) PS-CFP2 (fluorescence donor) and phiYFP (fluorescence acceptor). Cleavage of the substrate linker leads to physical separation of two fluorophores and to decrease in phiYFP fluorescence. Application of the developed assay allows us to rapidly perform end–point and kinetic measurement of LF activity. Kinetic constants for several substrates were calculated and comparative analysis of kinetic parameters of LF-mediated substrate proteolysis was carried out. We assume that the technique described can be used for the *in vitro* high throughput screening (HTS) of potential LF protease substrates and inhibitors.

Introduction

Anthrax toxin belongs to the family of binary toxins. It consists of Protective Antigen (PA), the single receptor-binding moiety that mediates internalization into target cells and two effector moieties, edema factor (EF) and lethal factor (LF). Lethal Toxin (LeTx), a complex of PA and LF, is the primary cause of toxic shock, no-return intoxication state and mortality in anthrax. LF is Zn-dependent metalloprotease cleaving MAP kinases thus disabling host immune response to anthrax bacilli. It is likely that LF also cleaves hitherto unknown cellular targets causing cell death. In recent years much effort has been put in discovery of small-molecule LF inhibitors that can serve as drug candidates for treatment of anthrax intoxication. Development of HTS assay for LF inhibitor screen requires sensitive and inexpensive analyte. Since LF is relatively inefficient protease, it requires long synthetic peptide substrates for efficient cleavage. Several peptide substrates modified by addition of fluorogenic or chromogenic dyes have been developed for monitoring of LF activity in HTS [1],[2]. Preparation of such substrates is not straightforward given the preferred LF recognition sequence that contains multiple positively charged amino acids [1], and resulting substrates are expensive especially if consider bulk quantities required for HTS.

Protein FRET is long considered as the alternative to peptide-based systems for detection of protease activity. Recently, new cyan fluorescent protein (PS-CFP2) was reported [3]. Excitation spectrum of PS-CFP2 is peaked at 402 nm. It has been shown that PS-CFP2 is good FRET-donor for the phiYFP protein (Evrogen, Russia). The emission of phiYFP is peaked at 538 nm, and fluorescence excitation at around 400 nm is low, yielding good spectral separation with PS-CFP2 excited with 400 nm light. Basing in these considerations, we chose PS-CFP2 (enhanced PS-CFP2 version, Evrogen) and phiYFP as a FRET-pair for LF protease activity detection. We engineered series of fusions between PS-CFP2 and phi-YFP, interconnected with the described LF peptide substrates and tested them in the LF cleavage assay.

Materials and Methods

DNA coding for LF domains 2-4 (LF24) was amplified from DNA of Bacillus anthracis Sterne strain by polymerase chain reaction and cloned into pET-based bacterial expression vector downstream to Gluthatione-S-transferase (GST) gene. The GST-LF24 fusion was expressed in Bl21(DE3) E. coli strain and purified at glutathione–Sepharose column.

Cloning vector, pCPhi based on the pQE30 backbone (QIAgen) for expression of the His6-tagged PS-CFP2 - phi-YFP FRET pair has been prepared. Vector sequence coding for the fused fluorescent proteins was splitted by 48 bp (16 amino acids) linker with different Lf substrate sequences. Fusion proteins were produced in soluble form in the BL21 E. coli strain transformed by pCPhi with inserted LF substrate sequences. After cell growing at 37 degree until OD (600)=0,6, cell culture was incubated at room temperature during 24 hours for fluorescent proteins chromophore maturation. Single-step purification of the FRET fusion was done by IMAC.

LF activity assay was carried out in 96-well black micoplates using TECAN Genious microplate fluorimeter set for excitation at 405 nm, and fluorescence detection at 535 nm. Substrate concentration varied from in two orders. In all experiments, LF concentration was fixed at 1 nM [4]

Results and Discussion

Three peptide substrates designed by combining semi-rational and artificial evolution approaches [4] were chosen as model substrates to estimate the applicability of FRET fusion protein system in the case of LF activity detection (see Table I).Selection of optimal conditions for LF-mediated substrate cleavage was performed. In particular, LF activity is inhibited at increased salt concentration. On the other hand, low-salt conditions support aggregation of FRET fusion proteins when the latter are taken at high concentration. Direct use of fluorescence readout, the following reaction conditions were chosen: 30mM Tris-HCl (pH 7,5), 100mM NaCl & 0,1 mM CaCl2. We next performed detection of LF inhibition by divalent metal ion scavenger, EGTA. Results are shown in Fig. 1.

CONTETNTS

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