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Plant biotechnology has emerged as an exciting area of plant sciences as it can create numerous opportunities to the manipulation and exploitation of the biological systems.

No other branch of biological sciences has developed in such a fast pace as plant biotechnology has. We are seeing the genes and genomes of a wide range of organisms being manipulated by use of new techniques for the benefit of man.

One of the key techniques in plant biotechnology is the genetic engineering involving gene transfer. Cell and plant tissue culture are the innovative breeding techniques applied to meet the increasing need for improved crop varieties. Tissue culture can significantly shorten the time, and lessen the labour and space requirements needed to produce new plant variety. The potential of cell and tissue culture for modern plant breeding can even be strengthen by induction of somatic and gametic embryogenesis. Plant breeders are striving to meet the challenge of increased production by developing plants with higher yield, resistant to pests, diseases and weeds and tolerant to various abiotic stresses. The impact of plant biotechnology on plant production and crop improvement is highly challenging in gradually becomes a reality. Plant biotechnology belongs to most powerful trends fighting for quality of life in 21 century.

The aim of the symposium on "Recent Advances in Plant Biotechnology" is to present the current developments and achievements in selected areas of plant biotechnology and communicate and discuss them.

Anna Preťová
Director of the IPGB SAS

Stará Lesná, June 10, 2007

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SIGNIFICANCE OF PLANT HORMONES FOR TISSUE CULTURES AND IMPORTANCE OF TISSUE CULTURES FOR HORMONE STUDIES

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Discovery of several plant hormones, namely of auxins and cytokinins, was directly connected with the effort to cultivate tissue cultures *in vitro* (almost eighty years ago). These two hormones, which are indispensable for cell division and growth, have become an important component of cultivation media for most tissue cultures. Manipulation of auxin/cytokinin ratio has been utilized to regulate the morphogenesis *in vitro*. Different auxins and cytokinins were found suitable for different species. Synthetic hormones, which are more stable and cheaper than the endogenous forms, have been often used. The other hormones have been applied much less frequently. Brassinosteroids were occasionally used to support cell division. Very important role has been played by abscisic acid in somatic embryogenesis, at the phase of embryo maturation. As far as ethylene is concerned, its removal after accumulation in tightly sealed containers was found beneficial in some cases.

The use of plant hormones has enabled establishment of tissue cultures from a wide range of species. *In vitro* techniques have been widely used for micropropagation, allowing the large-scale multiplication of the almost identical material. Important area of micropropagation is represented by somatic embryogenesis, which proved to be an important source of high quality plants, especially of trees. Optimal combinations of plant hormones are necessary in successive phases of embryo development.

Utilization of tissue cultures as a source of valuable substances did not fully meet the expectations, mainly due to the fact that the level of economically interesting secondary products was usually very low in undifferentiated tissues. This drawback was overcome either by tedious clone selection or by genetic modifications. *In vitro* cultivation is often crucial in order to get transformed material (especially when protoplast transformation has been chosen). In this way genetically modified plants of the first generation (enhanced tolerance to herbicides, pesticides etc.), the second generation (improved chemical composition, e.g. of lipid in vegetable oils) and the third generation (production of novel substances, e.g. vaccines) were achieved.

In the opposite way the tissue cultures became an important tool in plant hormone studies. Cell suspension cultures represent relatively simple and homogenous system for the elucidation of regulatory mechanisms in hormone metabolism. They enable to eliminate the effect of organ differentiation, various organ interactions as well as of environmental factors. Cell suspension cultures can be synchronized, which allows to follow the dynamics of hormone levels during cell cycle progression. In this way cytokinin elevation close to both cell cycle check-points was found. Using sequence for green fluorescent protein (GFP) gene constructs for many marker proteins were prepared. Using this system polar localization of PIN proteins (auxins efflux transporters) in BY-2 tobacco cell suspension was demonstrated. Auxin was found to stabilize these transporters in the membrane by suppression of their endocytosis. Recently the role of cytoskeleton in the positioning of PIN proteins has been evaluated using constructs with different fluorescent proteins. Apart of cell suspension cultures also somatic embryos were used for hormone studies. Using inhibitor of polar auxin transport the importance of tightly controlled auxin translocation at the early developmental phases was proved.

The above mentioned examples illustrate close relationship between plant hormones and tissue cultures.

OPTIMIZING THE MICROPROPAGATION PROTOCOL FOR THE ENDANGERED *Aloe polyphylla*: CAN *META*-TOPOLIN AND ITS DERIVATIVES SERVE AS REPLACEMENT FOR BENZYLADENINE AND ZEATIN?

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Benzyladenine (BA) is the most widely used cytokinin in the micropropagation industry due to its effectiveness and affordability. It, however, has disadvantages such as genetic alteration and abnormal growth in some plants. Naturally occurring zeatin on the other hand is not as widely used as BA and is far more expensive. The use of *meta*-topolin and its derivatives as alternatives to BA and zeatin, both of which frequently have negative effects in tissue culture was investigated. *In vitro* grown *Aloe polyphylla* Schonl. ex Pillans (an endangered medicinal and ornamental aloe) were cultured on full strength Murashige and Skoog basal medium with different concentrations of cytokinins and solidified with 1% Bacteriological Agar (Oxoid No. 1). *m*T was the preferred cytokinin both in terms of multiplication rate and rooting (Figures 1 and 2). The optimum concentration that induced regeneration and rooting was 5.0 µM (Figure 1 and 2). The problem of hyperhydricity was totally controlled (Table 1). Plants rooted spontaneously in multiplication medium, thus avoiding the extra rooting step of the protocol (Figure 2). More than 91% of the plants transferred to *ex vitro* conditions were successfully acclimatized. Significantly different growth response was also observed during growth *ex vitro*. *mT* treated plants produced larger shoot and root mass compared to other treatments (Table 2).

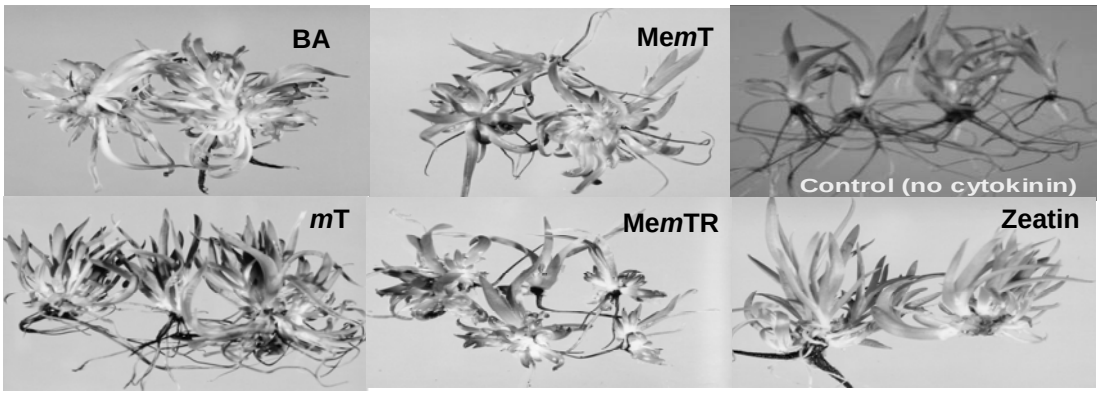

Figure 1. Effect of the selected optimum concentration (5 µM) on shoot and root growth and incidence of abnormality of *A. polyphylla*. Note the-*m*T treated plants with healthy shoot growth and numerous roots as opposed to the abnormal growth (BA) and failed rooting (zeatin)

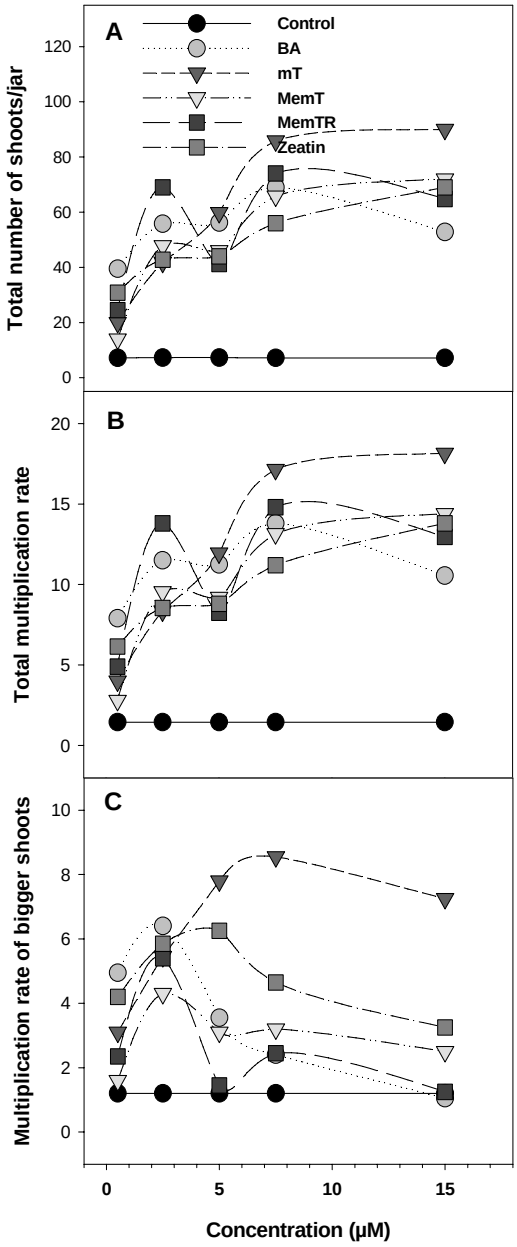

Figure 2. The effect of the type and concentration of cytokinin on shoot multiplication (A), total multiplication rate (B) and multiplication rate of shoots greater than 1.5 cm in length (C). Note that *meta*-topolin gave more shoots per jar and a larger number of shoots big enough for acclimatization. The 5 µM concentration was selected as an optimum concentration due to the good balance between shoot and root growth

Table 1. Effect of different types and concentrations of cytokinins on percentage of hyperhydric shoots (HS) and total number of shoots (TNS) *A. polyphylla*

Treatments	Cytokinin concentration (µM)									
	0.5		2.5		5.0		7.5		15.0	
	TNS	%HS	TNS	%HS	TNS	%HS	TNS	%HS	TNS	%HS
Control	7.3±1.31	0	7.3±1.31	0	7.3±1.31	0	7.3±1.31	0	7.3±1.31	0
BA	39.5±2.83	2.53	55.8±4.06	36.74	56.2±4.6	21.8	69.0±13.8	80	52.8±7.87	85.23
*m*T	20.0±4.34	0	41.8±2.15	0	59.8±2.43	0	85.8±7.92	25.64	90.8±10.4	58.6
Me*m*T	14.0±5.8	5.35	47.8±4.11	0	46.0±7.83	0	65.8±8.84	13.22	72.0±13.45	33.33
Me*m*TR	24.5±6.79	1.02	69.0±7.25	0	41.2±6.56	3.64	74.0±12.9	5	64.8±6.45	37.35
Zeatin	30.8±2.32	7.31	42.8±6.15	7.01	44.0±1.15	5.11	56.0±7.82	41.43	69.0±4.08	56.52
LSD (5%)	11.35		15		14.92		26.69		27.02	

Table 2. Effect of the different cytokinins tested on *ex vitro* growth. Fresh weight of ten (random) fully acclimatized (two-month-old) plants per treatment was used for this analysis. Only those treatments which produced plantlets with both shoots and roots in multiplication media were considered

Cytokinin concentration (µM)	Mean fresh weight (g)	
	Shoot	Root
2.5 BA	5.9±1.37	0.56±0.12
2.5 *m*T	19.3±2.83	1.75±0.26
2.5 zeatin	13.2±2.23	1.00±0.14
5.0 *m*T	23.6±7.03	1.66±0.44
5.0 zeatin	14.9±2.65	0.82±0.17
LSD (5%)	10.87	0.726

EFFECTS OF VARIOUS GROWTH REGULATORS ON OILSEED RAPE (*Brassica napus* L.) CALLUS FORMATION

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The object of the study was to explore the influence of various growth regulators on callus formation of oilseed rape (*Brassica napus* L.*)* of two different varieties ('Auksiai' and 'Vasariai'). Several explants from *in vitro* seedlings were cultured on Murashige &Skoog medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxyaceticacid (2,4-D) and 6-benzylaminopurine (BAP) ranging from 0.2-2.0 $mg.l^{-1}$.The efficiency of callus formation from explants of hypocotyls, leaves, roots, meristems was examined. It was noted that callus formation from hypocotyls and leaves of oilseed rape ('Auksiai','Vasariai') was higher on MS medium supplemented with 1.5 and 2.0 $mg.l^{-1}$ 2,4-D. The most effective callus induction was on MS medium with 1.5 $mg.l^{-1}$ 2,4-D from root explants. The formation of callus was increased significantly on MS medium with 2.0 $mg.l^{-1}$ BAP and 0.2 $mg.l^{-1}$ 2,4-D. On this medium the weight of callus was higher independently from explant type in comparison with MS medium with various 2,4-D concentrations. The callus induction depended on genotype, explant type and hormonal treatment. Callus cultures of oilseed rape were reported as suitable for extraction of glucosinolates (Poulsen, 1996).

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POSSIBLE PATHWAYS OF GIBBERELLIN–INDUCED DECLINE OF VIABILITY IN ISOLATED LARCH (*Larix sp.*) SHOOTS

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The development of larch (*Larix* sp.) short shoots *in vitro* was investigated using isolated axillary buds. The explants were collected from two 35-year old larch trees possessing different genotypes: European larch (*Larix decidua* Mill.) and hybrid larch (*Larix kaempheri* Carr. x *Larix decidua* Mill.) derived after crossing between Japanese larch and European larch. Isolated buds were planted onto MS nutrient medium supplied with distinct plant growth regulators. The negative effect of some kinds of plant growth regulators was estimated. Gibberellins (GA_3 and $GA_{4/7}$) had strong negative effect on the viability of isolated shoot primordia. Negative influence of auxin, though in less extensive rate, was also estimated. The explants of one genotype (hybrid larch) were far more resistant to these plant growth regulators than the explants collected from another (European larch). The certain role of developing primordia of axial needles in stimulation of chlorophyll loss was confirmed in European larch explants. Cytokinin zeatin when supplied to the nutrient medium together with gibberellin strongly promoted the negative effect of gibberellin (but this effect was clear only in European larch explants). It was also estimated that isolated larch shoots can act on new-planted explants from a distance if they share the same space for gas interchange (*in vitro*). This effect depended on the origin and previous treatment (with plant growth regulators) of these shoots while they developed from isolated axillary buds. Short shoots previously treated with auxin or gibberellin were significantly more sufficient inducing needle browning on newly developing shoots that shared the common space for gas interchange. The possible role of gaseous plant growth regulator ethylene is under discussion.

CHARACTERIZATION AND CONTROL OF SHOOT TIP NECROSIS IN MICROPROPAGATED *Harpagophytum procumbens*

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A study was made to characterize and control the factors governing shoot tip necrosis in micropropagated *Harpagophytum procumbens* [(Burch) de Candolle ex]. Components of *in vitro* plant growth and development such as media type, nutrients (Ca and B), sugars and plant growth regulators were investigated. Results of the experiments revealed that Murashige and Skoog (MS) media was more effective than woody plant media (WPM) for multiplication and growth (Table 1). Doubling the concentration of Ca and B independently in the MS media reduced the incidence of shoot necrosis. At an elevated concentration, B caused abnormal growth. However, when both Ca and B were used at double concentrations simultaneously, they resulted in retardation of growth (Table 2). In contrast to most reports, the use of cytokinins aggravated shoot necrosis. *Meta*-topolin riboside (*m*TR) was the preferred cytokinin in stimulating growth and controlling shoot necrosis (Table 3). The problem of necrosis was eliminated by transferring plants to cytokinin free rooting media (half strength MS + 2.5 μM IAA) when the first symptom of necrosis was observed in plants growing in multiplication medium. Accumulation of callus-like tissue at the base of the plants during multiplication prevented rooting. Higher mass of this callus-like tissue (Figure 1) was recorded with plants treated with benzyladenine (BA) compared to *m*TR. Trimming away this callus-like tissue completely was a prerequisite for rooting to occur.

Table 1. Effect of media composition and strength of media on shoot necrosis

Media	Average number of shoots/explant	Necrotic shoots (%)	Average shoot length (cm)
MS			
Full-strength	1.68 ± 0.09^{a}	88^{a}	5.08 ± 0.09^{a}
Half-strength	1.59 ± 0.07^{a}	29^{b}	5.08 ± 0.10^{a}
Quarter-strength	1.60 ± 0.07^{a}	14^{c}	3.26 ± 0.08^{b}
NB			
Full-strength	1.64 ± 0.07^{a}	90^{a}	3.69 ± 0.09^{b}
Half-strength	1.62 ± 0.06^{a}	27^{b}	1.53 ± 0.05^{c}
Quarter-strength	1.64 ± 0.07^{a}	26^{b}	*
WPM			
Full-strength	1.49 ± 0.05^{b}	86^{a}	3.31 ± 0.16^{b}
Half-strength	1.43 ± 0.04^{b}	27^{b}	*
Quarter-strength	1.23 ± 0.06^{c}	28^{b}	*

** shoot buds failed to attain measurable length*

Table 3. Effect of cytokinins and combination of cytokinins and IAA on growth of *H. procumbens*

Treatment (µM)	Shoot length (cm)	No. of branches	NNoST	NNeST	Fresh weight (g)	Necrotic shoots (%)
Control	3.03±0.24^{ab}	1.5±0.16^{c}	1.5±0.16^{bc}	0 ^{c}	0.14±0.02^{cd}	0
5 *m*T	4.05±0.16^{ab}	5.9±0.84^{a}	3.8±0.63^{a}	2.1±0.46^{b}	0.99±0.22^{bc}	35.59
5 *m*TR	4.00±0.27^{ab}	4.4±0.43^{b}	3.3±0.32^{a}	1.1±0.22^{bc}	0.56±0.07^{c}	25
5 BA	4.81±0.35^{a}	4.3±0.47^{b}	2.9±0.48^{ab}	1.4±0.29^{bc}	1.69±0.32^{ab}	32.56
5 BA + IAA	3.43±0.17^{ab}	6.2±0.66^{a}	2.4±0.62^{ab}	3.8±0.56^{a}	2.26±0.13^{a}	61.29
5 *m*T + IAA	3.75±0.14^{ab}	6.9±0.44^{a}	2.6±0.57^{ab}	4.3±0.58^{a}	1.39±0.15^{b}	62.32
5 *m*TR+ IAA	3.60±0.43^{ab}	5.3±0.43^{ab}	3.3±0.58^{a}	2±0.37^{b}	1.31±0.09^{b}	37.73
10 BA + IAA	6.78±2.9^{a}	4.2±0.46^{b}	2.1±0.36^{ab}	2.1±0.49^{b}	2.03±0.18^{a}	50
10 *m*T + IAA	3.35±0.16^{ab}	7.2±0.59^{a}	4.3±0.39^{a}	2.9±0.74^{ab}	2.1±0.19^{a}	40.28
10 *m*TR + IAA	2.90±0.22^{ab}	6.9±0.48^{a}	4.6±0.55^{a}	2.3±0.37^{b}	2.45±0.36^{a}	33.33

Note that NNoST = number of normal shoot tip; NNeST = number of necrotic shoot tip; mT = meta-topolin.

Values represent mean ± standard error for all tables. Values followed by the same letter in each column are not significantly different ($p \leq 0.05$).

Table 2. Effect of B and Ca on shoot-tip necrosis (STN)

Treatments (mM)	Mean No. of STN per jar
Control (MS)*	4.8±2.5^{b}
6 Ca	3.2±0.66^{b}
9 Ca	1.6±1.3^{bc}
12 Ca	1.4±0.91^{bc}
15 Ca	1.0±0.62^{bc}
0.2 B	4.4±0.91^{b}
0.3 B	3.2±0.90^{b}
0.4 B	0.4±0.39^{bc}
0.5 B	0.0b^{c}
0.2 B + 6 Ca	8.0±1.12^{a}
0.3 B + 9 Ca	4.0±1.34^{b}
0.4 B + 15 Ca	1.2±0.37^{bc}
0.5 B + 15 Ca	1.8±0.48^{b}

** 3 mM Ca and 0.1 mM B*

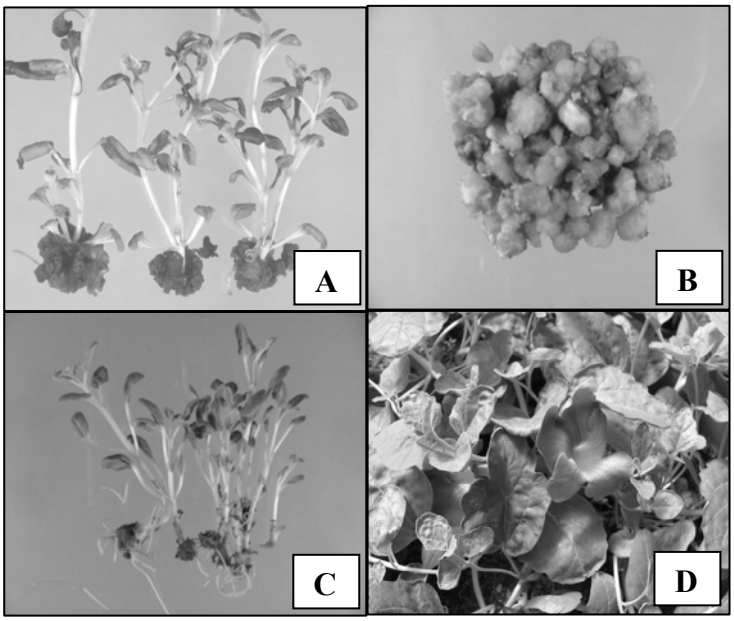

Figure 1. *In vitro* grown *H. procumbens* (**A**) necrotic symptom and development of basal callus; (**B**) trimmed basal callus; (**C**) rooted plants ready for acclimatization and (**D**) fully acclimatized and greenhouse-grown plants

SECRETION OF CHITINASE IN LIQUID CULTURES OF FLAX IS CONNECTED WITH THEIR MORPHOGENIC RESPONSE

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Despite the regeneration via somatic embryogenesis recently provides very effective method for propagation of plenty of plant species, reports on flax somatic embryogenesis are still scare (Preťová and Williams, 1986; Cunha and Ferreira, 1996; Tejavathi *et al*., 2000). Moreover, fully optimized protocols for initiation and regeneration of flax somatic embryos are still lacking.

It has been proved that many signal molecules like arabinogalactans, lipo-oligosacharides peptides, pathogenesis-related proteins (namely chitinases), secreted into culture medium by cells and multicellular structures, can influence the process of somatic embryogenesis (van Hengel, 1998; Kasprzewska, 2003; Matthys-Rochon, 2005). The conditioned medium of embryogenic cultures is,a rich source of endogenous molecules promoting the formation of somatic embryos (Maës *et al*., 1997). Moreover, a direct correlation was established between flax embryo-like morphogenesis and a set of extracellular proteins strongly bound to the cell walls (Roger *et al*., 1998).

The aim of our experiments was to initiate flax somatic embryogenesis through liquid culture, as well as to compare the effect of conditioned and non-conditioned culture media on morphogenesis and growth of flax suspension culture.

Two cultivars of flax (*Linum usitatissimum* L.), Belinka (samples No. 1- 4) and Jitka (No. 5 - 8), were used in our experiments. To establish the suspension cultures we used flax calli (8 samples differing in genotypes, primary explants used for initiation, and growth regulators in culture media) growing on solid MS medium, supplemented with 1 $mg.l^{-1}$ 2,4-D or combination of 2,4-D (1 $mg.l^{-1}$) and BAP (0.25 $mg.l^{-1}$). The obtained white and friable calli, induced primary from 6-day-old cotyledons and hypocotyl segments as well as from immature zygotyc embryos (Petrovska *et al*., 2007), were then transferred into the liquid MS medium of the same composition. Four samples (number 1, 2, 5, 6) were conditioned by adding 500-3000 µl of DCR liquid medium from highly embryogenic *Pinus nigra* (cell lines E140 and E146) suspension cultures (Salaj *et al*., 2007), and four samples (3, 4, 7, 8) served as a non-conditioned control. All cultures were cultivated at 21 ^{o}C in the dark on the rotary shaker at 100 rpm. The subculture was carried out every two weeks.

Detection of chitinase activity on 12.5% (w/v) SDS-PAGE slab gels with glycol chitin (0.01%) as an enzyme substrate, was described in details earlier (Matušíková *et al*., 2005). For histological observations we used paraffin sections (5 µm thick) stained with 1% toluidine blue (Salaj *et al*., 2005).

The growth of flax-suspension culture (expressed as a sedimented cell volume - SCV) induced from leaves, hypocotyls and immature zygotic embryos, was compared in both conditioned (with *P. nigra* liquid culture) and non-conditioned media. The calli in non-conditioned media were mostly compact and firm (except for sample No.7) while in conditioned ones were soft and no significant differences in SCV were found. However,

no embryo-like morphological response has been found in latter medium, suggesting, the conditioning had no morphogenic effect. Biochemical analyses have shown chitinase activitity in both conditioned as well as in non-conditioned media. In the sample No.7 (non-conditioned), which has also embryo-like morphological response, the presence of approx. 25 kDa flax chitinase has been found, indicating, this 25 kDa flax chitinase can play some role in the formation of flax embryo-like structures.

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OXIDATIVE MARKERS OF RHIZOGENESIS IN TISSUE CULTURE OF *Mesembryanthemum crystallinum* L.

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Direct and indirect root regeneration in culture of seedling-derived hypocotyls of *M. crystallinum* was obtained. Direct rhizogenesis occurred within 10-14 days on modified MS medium (Libik *et al*., 2005) with 1 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ kinetin (DK medium) (Fig. 1, Tab. 1). Next hypocotyls started to produce callus with no visible roots. After subculture of such calluses on modified MS with 0.2 $mg.l^{-1}$ kinetin (K medium) as the sole growth regulator abundant root formation occurred within 4 weeks (Fig. 2, Tab. 1). Notably, when hypocotyls were maintained directly on K medium, without pre-culture on DK rhizogenesis was not observed. Histological observations showed that the first cell divisions giving rise to roots occurred after 1 day and on day 5th clusters of meristematic cells inside the hypocotyl could be found (Fig. 3). Following the next 2-5 days roots with well developed root cap protruded over hypocotyl epidermis (Fig. 4). With continued culture parenchymatous callus with clumps of meristematic cells was obtained. After 4 weeks of subculture on K medium from these clusters numerous roots were formed. Culture on DK and K medium was accompanied by changes in endogenous H_2O_2 content and in activities of some antioxidant enzymes in explants. Several forms of superoxide dismutase (SOD) in material studied was revealed: MnSOD, FeSOD and Cu/ZnSOD. Cu/ZnSOD and FeSOD showed similar pattern of activity in all of the material, while on day 5th on DK the additional band of MnSODII occurred. It could be also detected in calluses showing rhizogenesis on K medium. Interestingly in the explants maintained directly on K medium, where roots were not induced, the band of MnSODII was not found (Fig. 4). MnSODII has already been identified in roots and callus of *M. crystallinum* and its expression has been suggested to be related to heterotrophic nature of root and cultured tissue (Ślesak and Miszalski, 2003; Libik *et al*., 2005). In order to verify whether the occurrence of MnSODII is related to root formation we used a tissue transfer experiment (Christianson and Warnick, 1983) which allow to discriminate the developmental events of induction and determination during organogenesis *in vitro*. The results showed that the induction of determination for rhizogenesis occurred on the 5th day of culture on DK medium. Since at this time the activity of MnSODII occurred we speculate that this specific isoform of SOD can be regarded as a marker of rhizogenic determination in *M. crystallinum*. Additionally, the measurement of maximum photochemical efficiency of photosystem II (F_v/F_m) of chlorophyll *a* confirmed an autotrophic nature of the cultured tissues, excluding the possible relation between the expression of MnSODII and heterotrophic metabolism. The observed changes in SOD activities as well as in activities of catalase and guaiacol peroxidase in relation to endogenous H_2O_2 content and their possible involvement in root regeneration in culture of *M. crystallinum* are discussed.

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Table 1. Qualitative data of rhizogenesis in culture of *M. crystallinum*. Data were scored after 14 days of culture on DK medium (direct rhizogenesis) and after 4 weeks of subculture on K medium (indirect rhizogenesis).

Direct rhizogenesis		Indirect rhizogenesis	
%	mean no. of roots/explant	%	mean no. of roots/callus
65	6.3 ± 1.6	97	45.5 ± 17.1

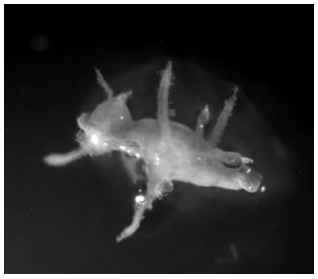

Figure1. Direct rhizogenesis on DK medium

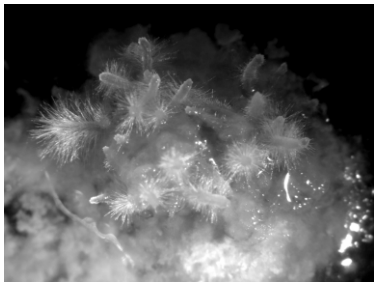

Figure 2. Indirect rhizogenesis after transfer from DK to K medium

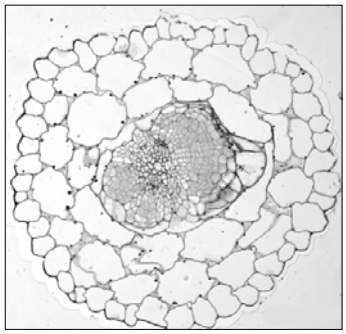

Figure 3. Section of hypocotyl after 5 days on DK medium

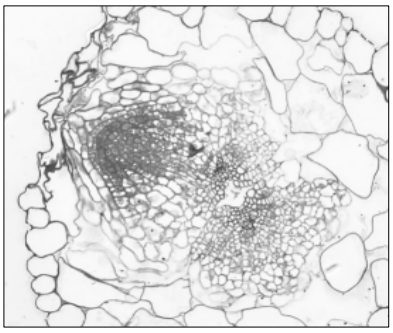

Figure 4. Section of hypocotyl after 7 days on DK medium

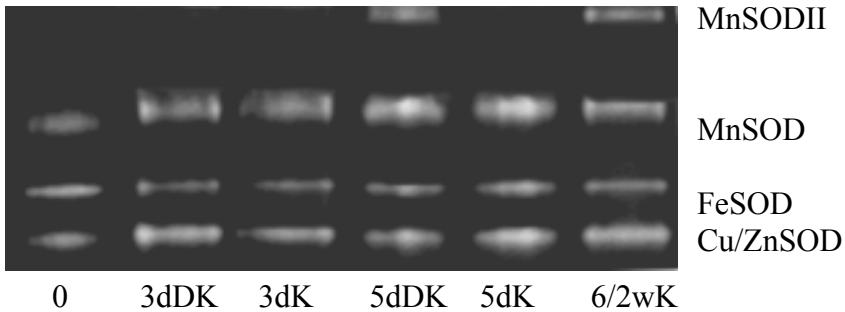

Figure 5. SOD pattern of cultured explants: 0-explants before culture, 3d - three days on DK and K medium; 5d - five days on DK and K medium, 6/2w - six weeks on DK and 2 weeks on K medium (callus culture)

MICROPROPAGATION OF APPLE CULTIVARS IDARED, SAMPION AND PEAR CULTIVARS LUCASOVA AND MAX RED BARTLETT

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The use of *in vitro* culture approaches constitutes an important tool for multiplication of selected cultivars. The objective of this study was to develop a method for rapid *in vitro* shoot multiplication of two apple and two pear cultivars. Five proliferation MS media containing 1, 2 and 4 mg.l^{-1} BAP or 0.5 and 1 mg.l^{-1} TDZ were tested. For the four cultivars, the effect of two growth regulators in different concentrations on proliferation, callus formation and shoot morphology is shown. Proliferation rates varied depending on the genotype and medium used. The highest multiplication rate was obtained for pear cultivar ´Lucasova´ that produced 5.0 ± 0.2 shoots (longer than 10 mm) on MS medium containing 4 mg.l^{-1} BAP. The lowest multiplication rate was obtained for apple cultivar ´Idared´ producing only 1.2 ± 0.1 shoots on MS medium containing 1 mg.l^{-1} BAP. Abundant callus formation at the base of explants was observed in all variants on the media containing TDZ.

Acknowledgement
This work was supported by Ministry of Agriculture of the Czech Republic (project NAZV 1B44051).

FACTORS IMPORTANT FOR EFFECTIVE ANDROGENESIS INDUCTION IN ISOLATED MICROSPORE CULTURE OF *Triticale* (*x Triticosecale* WITTM.)

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Isolated microspore culture (IMC) can be used as a convenient system in crop improvement, genetic manipulations and in many areas of basic research. It allows chieving total homozygosity in one generation and can be performed at any step of a breeding program. As compared with traditional breeding, this system can save time, costs and labor expenditures. The development of microspore or pollen grain into embryo-like structure (ELS), the process called 'androgenesis' mimics the zygotic embryogenesis *in vivo.* Therefore, IMC can be used as an ideal model for studies of embryogenesis and other aspects of plant development. Microspores are also ideal targets for genetic transformation, transgenic studies and mutagenesis induction. Produced haploids or doubled haploid (DH) lines are frequently used in genome mapping and other genomic studies. However, incorporation of IMC into breeding programmes or other studies is limited by the fact that in many cases the efficiency of the androgenesis is not satisfactory. This problem concerns also triticale, thus the development of an efficient method of IMC is appreciated.

The studies were carried out on two Polish spring cultivars of triticale ('Mieszko' and 'Wanad', *X Triticosecale* Wittm.), significantly differing in their response to androgenic induction and plant regeneration. In a 4-years sequence of experiments, every stage of androgenesis induction and ELS development starting from spike pretreatment and anther preculture, through *in vitro* microspore culture, ending at plant regeneration stage was examined. Changes of some bio-chemical and bio-physical parameters connected to androgenic induction (among other: the content of abscisic acid, the activity of antioxidative enzymes, the level of metabolic activity) were studied. Several factors important for the high efficiency of the process were identified. As the result, the optimized procedure for triticale IMC was established and introduced into practice at the Institute of Plant Physiology PAS in Kraków, Poland.

BIOENERGETICS OF CONIFEROUS SOMATIC EMBRYOGENESIS

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Somatic embryogenesis provides an ideal experimental model for studying the bioenergetic events that occur during the onset of developmental steps leading to the plant regeneration. In the present study, the bioenergetic processes of *Picea abies* and *Abies cephalonica* somatic embryogenesis were examined using two different approaches. The first concerns the isolation and characterization of mitochondria from embryogenic tissues of *P. abies* and *A. cephalonica.* The second focuses on a detailed characterization of biochemical markers (cellular ATP and glucose-6-phosphate) during proliferation and maturation of *A. cephalonica* treated with humic substances.

The role of mitochondria during the process of organogenesis or somatic embryogenesis has been neglected for a long time. Papers dealing with isolation and characterization of mitochondria from Gymnosperms are rare. In the present study, a valuable method for isolatation of crude and purified mitochondria from embryogenic cell masses (ECMs) of *P. abies* and *A. cephalonica* cultivated on proliferation media is described. The ECMs of both species were characterized according to the proportion of different developmental stages of proembryogenic cell masses (PEM): PEM I, PEM II, PEM III and filamentous somatic embryos. Both crude and Percoll-purified mitochondrial fractions were collected and analyzed for sub-cellular contamination, oxygen uptake, mitochondrial electrical potential (m$\Delta\Psi$) formation and inner membrane integrity. Crude mitochondria *per se* showed acceptable values of the major bioenergetic parameters (e.g. R.C.R., membrane integrity, m$\Delta\Psi$ value), but contained, in some cases, appreciable levels of membrane contamination. After purification of crude mitochondria by a discontinuous Percoll gradient three fractions, all displaying mitochondrial activities, were obtained. The presence of a K^+_{ATP} channel in crude mitochondria was examined as potassium-inward activity (KCl-induced m$\Delta\Psi$ dissipation after malate plus glutamate energization) and spontaneous potassium-outward activity (evaluated as CsA-dependent m$\Delta\Psi$ building). The mitochondrial K^+_{ATP} channel activity was further examined in the 23/40 mitochondrial fraction of both species. The KCl-induced dissipation was still evident in these purified (energized) mitochondria, although the magnitude was smaller than that recovered in crude fraction from both species. Conversely, in de-energized mitochondria, swelling (K^+-outward flux) reached a higher level with respect to that monitored in crude mitochondria. These differences can be related to the presence of a higher amount of well-coupled and intact mitochondria in the purified fraction with respect to the crude ones.

The isolation and characterization of mitochondria from embryogenic cell masses of *P. abies* and *A. cephalonica* have shown that these organelles are similar to those found in Angiosperms (Pastore *et al.,* 1999; Petrussa *et al.*, 2001). They possess a K^+_{ATP} channel with similar characteristics and this is a prerequisite for conducting further advanced biochemical studies about their involvement in responses to biotic stress, cell adaptation to drought and programmed cell death (Pastore *et al.*, 2007; Vianello *et al*., 2007).

Humic substances (HS), as supramolecular associations of heterogeneous molecules deriving from microbial degradation of natural organic materials, are ubiquitous in water, soil, and sediments (Piccolo, 2001). They exhibit positive effects on some physiological aspects of plant growth and metabolism (Nardi *et al.*, 2002). The second series of experiments was conducted to study the effect of HS on embryogenic cell masses of *A. cephalonica*. ECMs were treated with 1 mg/Petri plate humic or fulvic acids at regular intervals (3, 7, 14 and 21 days). The effects of HS on the proliferation rate, consecutive developmental stages of PEMs, level of ATP and glucose 6-P were evaluated. Humic acids had significant effects just on the biochemical parameters evaluated, such as the level of ATP and glucose 6-P, which were increased on days 3, 7 and 14. Fulvic acids were able to enhance, significantly, the proliferation rate, especially during the early stages, and to affect the proportion of PEMs, in particular enhancing the percentage of PEM III at day 14. Furthermore, fulvic acids induced an increase of cellular ATP concentration, but not of glucose 6-P. These results indicate that HS might play an important role in improving the proliferation of PEM, thus affecting the subsequent maturation process of *A. cephalonica*. The effect of HS was also studied on maturation media supplemented with different concentrations of ABA and polyethylene glycol.

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STRUCTURAL ASPECTS OF SOMATIC EMBRYOGENESIS IN ZYGOTIC EMBRYO CULTURE OF *Trifolium nigrescens* VIV.

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Somatic embryogenesis (SE) can be used as a model system for studying the mechanisms underlying plant growth and differentiation. In *Trifolium nigrescens* Viv. SE has already been observed, but its frequency as well as the efficiency of plant recovery was relatively low (Konieczny, 1995). In this study we present a new method to obtain an efficient SE using mature zygotic embryos as explants.

Zygotic embryos (about 1 mm) were cultured on MS medium supplemented with 0.5 $mg.l^{-1}$ NAA and 2 $mg.l\ l^{-1}$ 2iP for 28 days. After 3-6 days the explants became swollen and in the hypocotyls region the yellow callus occurred. Following 3-5 days callus covered a whole explants which was accompanied by formation of somatic embryos (Fig.1). Most of the embryoids were produced in groups, randomly distributed through the callus. Finally, after 28 days of culture about 60% of the explants produced SE with an average frequency of 9-11/callus. Embryoids at the torpedo stage or older were harvested from maternal tissue and transferred on half-strength MS for germination. About 25% of somatic embryos regenerated to flowering plants (Fig. 2). Histological analysis revealed, that no cell division in the embryos before explantation was observed. The onset of regeneration occurred after 3 days of culture. At this time intense mitotic activity in outer cortex led to the formation of groups of meristematic cells with large, centrally positioned nuclei. The specific arrangement of these groups within the hypocotyls indicated their origin from single cortical cell of the embryo. Within 3-5 days of culture the cell divisions in cotyledons were less intense than within hypocotyls and limited to the parenchyma of adaxial surface of the organ. After 7 days of culture the periclinal divisions of epidermal cells of both cotyledons and hypocotyls together with extended mitotic activity of subtending parenchyma led to disruption of epidermis and outgrowth of the callus over explant surface. Initially callus consisted of large parenchymatous cells, among which small densely stained cells of meristematic nature could be found. From these meristematic cells round-shaped structures, resembling somatic embryos at the globular stage arose (Fig.3). The first bipolar somatic embryos with clearly distinguishable root and shoot pool could be observed on the 9th day of culture. However most of the embryoids obtained in our experiment displayed several morphological abnormalities such as: fusion of embryo axes, elongated hypocotyls (trumpet type of embryoid), cotyledons fusion, additional cotyledons or only one cotyledon (Fig.4). Very often a precocious germination of somatic embryos was noted. The observed relatively low conversion rate of somatic embryos into plants seems to be clearly related to various morphological abnormalities induced under described *in vitro* conditions. Observations in SEM revealed, that early stages of embryoid formation were accompanied by a conspicuous fibrillar network coating the surface cells of globular and heart-shaped embryoids (Fig.6). This so called extracellular matrix surface network (ECMSN) was also observed on some callus cells as coarse strands and fibrils connecting neighboring cells. Occasionally ECMSN as a compact layer coating the large area of callus was also observed.

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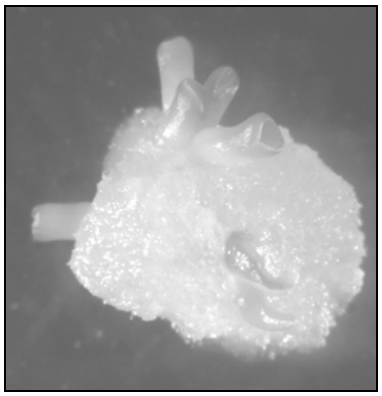

Figure 1. Somatic embryos on callus after two weeks of culture

Figure 2. Regenerated plants

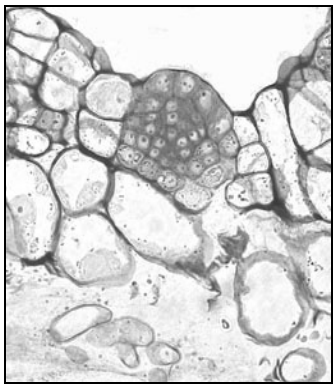

Figure 3. Globular embryo-like structure

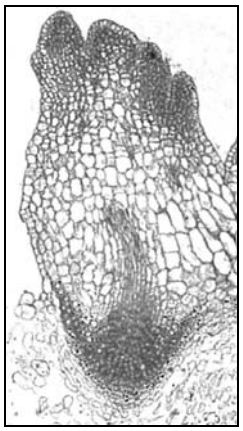

Figure 4. Somatic embryo with poorly developed cotyledons

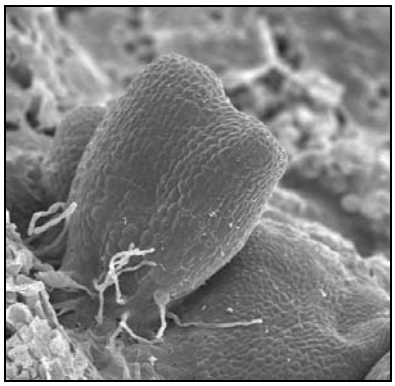

Figure 5. Heart-shaped embryoid observed in SEM

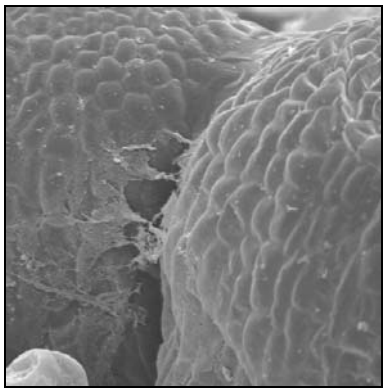

Figure 6. Fibrillar ECMSN joining surface cells of embryoids

USE OF MITOTIC POLYPLOIDIZATION AND SOMATIC HYBRIDIZATION FOR CREATING OF NEW BREEDING MATERIALS IN *Solanum* GENUS

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Major breeding aim is increasing tolerance to late blight in cultured potatoes considering that the potato late blight is the most serious potato disease caused by the pathogen *Phytophthora infestans* (Mont de Bary). The genes for resistance could be transferred by utilization of wild *Solanum* species. Polyploidizatization and somatic hybridization are very useful tools in plant breeding to overcome hybridization barriers.

Obtaining of breeding material using polyploidization was performed with colchicine and oryzaline application to nodal segments of wild potato *S. berthaultii*, *S. bulbocastanum*, *S. pinnatisectum*, *S. verrucosum* and dihaploids of *S. tuberosum*. Regenerants were evaluated by flow-cytometry and derived tetraploids were used in crossing with *Solanum tuberosum*.

Somatic hybridization was done by an electrofusion of mesophyll protoplasts. *Solanum tuberosum* (cv. Bintje, Ditta, Keřkovský rohlíček, Kordoba, Magda; dihaploid 243 and 299), *S. berthaultii*, *S. bulbocastanum*, *S. pinnatisectum* and *S. verrucosum* were used. There were obtained calli and plant regenerants from following electrofusion combinations: *S. bulbocastanum* + Bintje, *S. bulbocatanum* + Ditta , *S. bulbocastanum* + Magda, *S. bulbocastanum* + Keřkovský rohlíček, *S. bulbocastanum* + Kordoba, *S. bulbocastanum* + 243, *S. pinnatisectum* + Ditta, *S. pinnatisectum* + Keřkovský rohlíček, *S. pinnatisectum* + Bintje, *S. pinnatisectum* + 299. Regenerated plants were assessed by flow-cytometry and a RAPD method. We obtained somatic hybrids in several combinations. The somatic hybrids are morphological evaluated.

Acknovledgement

This work was supported by project NAZV QF 4133.

IN VITRO REGENERATION OF PLANT AND INDUCTION OF FLOWERING IN *Capsicum frutescens* MILL.

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Capsicum frutescence Mill. is an important horticultural crop belonging to the family *Solanaceae*. Its fruits are extensively used as vegetables and spices (Bosland, 1994). Chilli is found to be highly recalcitrant and highly genotype specific (Ochoa-Alejo and Ramirez-Malagon, 2001). Application of cell and molecular biology techniques for genetic improvement of chilli are limited. The genetic manipulation of *Capsicum* has been unsuccessful and a big bottleneck for transferring the desired genes because of the difficulty in regenerating whole plants by tissue culture. Even the recent reports are confined only to the PEG mediated transformation of mesophyll cells in *C. annuum* (Jeon *et al*., 2007). In this context, the rapid organogenesis through direct shoot bud induction in two varieties of high pungent *Capsicum frutescens* was obtained from the decapitated seedling explants inoculated in inverted mode on to the shoot bud induction medium (SBIM) comprising MS salts, supplemented with 2-(N-morpholine) ethane sulphonic acid (MES), IAA and BA. Maximum of 30 and 45 shoot buds were induced in both KT-OC and BOX-RUB varieties of *C. frutescens* respectively. Presence of both gibberellic acid (GA_3) and silver nitrate ($AgNO_3$) in the shoot bud elongation medium (SBEM) is found to be crucial for the effective elongation of shoot buds into ~ 4.0 cm length shoots in 4-6 weeks. Apart from this, for the first time we have successfully established a novel pathway for rapid shoot regeneration from the proximal zone of the leaf of *Capsicum frutescens*, in which leaf segments were taken from *in vitro*-proliferated shoots of *C. frutescens* established in aseptic culture from shoot tips of field-grown plants. Benzyl amino purine (BAP) played a significant role in the induction of shoot buds without callusing. Shoot buds were induced directly in Murashige and Skoog's (MS) medium supplemented with BAP (10 $mg.l^{-1}$) + IAA (10 $mg.l^{-1}$) + $AgNO_3$ (10 µl) + MES (1.98 $g.l^{-1}$). In this medium 13-15 shoot buds differentiated from the proximal margin of the lower petiolar segment within 4 wk of incubation. Differentiated shoots grew well and proliferated in the MS medium having PAA (2$mg.l^{-1}$) + BAP (7 $mg.l^{-1}$) and 10 $mg.l^{-1}$ $AgNO_3$; subsequently these shoots were rooted on hormone free ½ strength MS medium in 4 weeks. Upon hardening in green house, the field survival rate was 70%. This *in vitro* derived plants were phenotypically and cytological normal, which may be of use in genetic transformation studies. *In vitro* floral induction and floral morphogenesis was also studied using inhibition of ethylene biosynthesis ($CoCl_2$) (Locke, 2000) and ethylene action ($AgNO_3$) (Beyer, 1976). Exogenous administration of $AgNO_3$ and $CoCl_2$ at a concentration of 30 µM resulted in the maximum tissue response in terms of shoot length and number of shoots after 45 days of culture on MS medium. Both Silver nitrate (40 µM) and Cobalt chloride (30 µM) influenced *in vitro* flowering after 25 and 45 days respectively. This is the first report on *in-vitro* flowering in *C. frutescens* (Indian patent pending. 764/DEL/2004). This study will be useful for pollen transformation using germ free *in vitro* flowers.

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POSTERS

DISTINCT BIOLOGICAL ACTIVITIES AND METABOLIC PATHWAYS OF *cis*- AND *trans*-ZEATINS IN PLANTS

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Cytokinins (CKs) play an important role as growth regulating compounds in plants. They affect numerous aspects of plant development and physiology including seed germination, de-etiolation, chloroplast differentiation, apical dominance, plant-pathogen interactions, flower and fruit development, and leaf senescence. The naturally occuring CKs are N^6-substituted adenine derivatives bearing an isoprenoid or aromatic side chain. CKs with the unsaturated isoprenoid side chain are by far the most prevalent in plants, in particular those with a *trans*-hydroxylated N^6-side chain, i.e. *trans*-zeatin (*t*Z) and its derivatives (Mok and Mok, 2001). A natural occurence of *cis*-zeatin (*c*Z)-type CKs in plants has also been known for years, when their presence as minor CK constituents has been attributed mainly to their origin from tRNA (Kamínek, 1974). With respect to rather low activities (Schmitz *et al*., 1972), *cis*-zeatins were viewed long-time as adjunct to their corresponding *trans*-isomers. However, the recent development and introduction of modern sophisticated methods for CK analysis revealed surprisingly high concentrations of *c*Z derivatives exceeding those of *t*Z in many plant species including mono- and dicotyledonous (e.g. Emery *et al*., 1998; Veach *et al*., 2003). This indicates that *c*Z-type CKs are more prevailing and probably more relevant to CK biology than previously thought, having unique functions in plant tissues and being synthesized in a distinct way compared to their corresponding *trans* isomers (Kasahara *et al*., 2004; Miyawaki *et al*., 2006). The recent finding of genes and enzymes specific for *c*Z-type CKs (Martin *et al*., 2001; Veach *et al*., 2003) as well as the demonstration of *c*Z receptor binding (Spíchal *et al*., 2004; Yonekura-Sakakibara *et al*., 2004) affirm such assumptions.

The aim of this study was to elucidate some aspects relating to biological activities and metabolism of *t*Z- and *c*Z-type CKs in oat (*Avena sativa* L. cv. Abel) and tobacco (*Nicotiana tabacum* L. cv. Samsun NN). Both oat and tobacco leaves were found to contain *c*Z derivatives in relatively high concentrations exceeding those of their *trans* counterparts with the prevalence of *c*Z-*O*-glucoside (*cZO*G) and *c*Z-*N*7-glucoside (*c*Z7G), respectively. In excised oat leaf segments, biological activities of *t*Z and *c*Z as well as their ribosides, *N*- and *O*-glucosides were tested in a chlorophyll retention bioassay. For all assayed CK forms, the *c*Z derivatives showed significantly lower (at least 5-fold) activities in inhibiting chlorophyll degradation in dark compared to their corresponding *trans* isomers. To acquire information about CK interconversions, the uptake and metabolic fate of [2-^{3}H]*c*Z and [2-^{3}H]*t*Z were investigated in detached oat and tobacco leaves by HPLC coupled to on-line radioactivity detector. In accordance with the prevailing occurence of endogenous *cZO*G in oat and *c*Z7G in tobacco, radiolabelled *cZO*G and *c*Z7G were also detected as predominant [2-^{3}H]*c*Z metabolites

during 2, 5, 8 and 24 h incubation in oat and tobacco isolated leaves, respectively. The application of [2-^{3}H]*tZ* led to the detection of a great number of CK forms with the prevalence of *tZ*-*N*-glucosides in both plant materials; in tobacco leaves a relatively high portion of radioactivity was apparently associated also with *tZ*-riboside-*O*-glucoside. Increasing formation of adenine and adenosine during incubation of oat and tobacco detached leaves indicated extensive degradation of both [2-^{3}H]*cZ* and [2-^{3}H]*tZ* by cytokinin oxidase/dehydrogenase (CKX). High CKX activity was confirmed in protein preparations from oat and tobacco by *in vitro* assays. In agreement with literature data (Armstrong, 1994), N^6-(Δ^2-isopentenyl)adenine was found to be the preferred CKX substrate for both oat and tobacco leaf enzyme. Although *cZ*-type CKs have been so far mostly reported as rather bad substrates for CKX (weaker than *trans*-zeatins), our enzymatic studies with [2-^{3}H]*cZ* and [2-^{3}H]*tZ* on oat and tobacco leaf enzymes revealed that *cZ* was degraded with respective velocities up to 2-fold and 4.5-fold higher than *tZ*.

With respect to the obtained data, a potential role of *cis*- and *trans*-zeatins in regulation of plant growth and development as well as in establishment and maintenance of CK homeostasis in plants will be discussed.

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AN ANALYSIS OF PLANT HORMONES BY LIQUID CHROMATOGRAPHY WITH UV/MS DETECTION

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Due to physiological regulation plants dispose of several basic groups of hormones as stimulative ones - auxins, cytokinins, brassinosteroids, gibberellins or inhibitive as jasmonic acid, abscisic acid as well others. To their separation and determination reversed phase high performance liquid chromatography coupled with various types of detectors can be used (Novak *et al*., 2003). There has been published plenty of papers reported on measuring only one group of plant hormones. Nevertheless co-action of various types of plant hormones are crucial to regulate biochemical pathways, thus, the more complex analysis of them is needed to better understanding not only of such pathways but also of affecting of a plant by stress factors including heavy metals (Barendse and Peeters, 1995; Weyers and Paterson, 2001). Besides this hormones need not to occur in their active form. Their metabolites such as glucosides, ribosides and others, which can served as deposits or can be degraded form of single hormone, have been identified (Kleczkowski and Schell, 1995). Based on the mentioned facts we aimed on optimizing experimental conditions to separate and determine not only plant hormones but also their metabolites by liquid chromatography coupled with UV-VIS/mass detector.

The mixture we analyzed was composed from trans-zeatin, cis-zeatin, zeatin riboside ane glucoside, aromatic cytokinins and their ribosides, natural and synthetic auxins as indolyl acetic acid or naphtylacetic acid, and synthetic cytokinins as benzyladenine or kinetin. We have optimized composition of mobile phase and its flow rate, column temperature, and gradient elution to reach low detection limits. The limits were estimated as units and/or tens nM for the target molecules. The optimized technique was subsequently utilized to analysis of plant samples.

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PROTOPLAST CULTURE OF CUCUMBER (*Cucumis sativus* L.)

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Cucumbers are attacked by various diseases (e.g. downy mildew - *Pseudoperonospora cubensis*) which affect crop yields. The breeding options are rather limited since this species can not be crossed with the wild species of the genus *Cucumis*, which possess resistance to some of the diseases. Plant protoplast culture can be used for somatic hybridization by fusing protoplasts of two different species (Carlson *et al*., 1972). The aim of the experiments was to improve the efficiency of the protoplast culture technique, i.e. increase the number of dividing cells which grow in microcalluses.

Seeds of *Cucumis sativus* (line 6514) were obtained from Research Institute of Crop Production, Gene Bank in Olomouc, CZ. Plants were grown on OK medium (MS with 20 $g.l^{-1}$ sucrose, 0.01$mg.l^{-1}$ IBA, 0.01$mg.l^{-1}$ BA, 20 $mg.l^{-1}$ ascorbic acid) in 23 °C. Leaves of 20-29 days old plants were cut into 1mm pieces and incubated in enzyme solution (1% Cellulase Onozuka R-10, 0.25% Macerozyme R-10 in PGly washing solution ([Debeaujon and Branchard, 1992]) in dark, 27 °C for 16hr. The protoplast suspension was purified by repeated centrifugation at 800 rpm. They were cultured in liquid LCM1 medium (NAA/2,4-D/BA 1.0/0.5/0.5 $mg.l^{-1}$; Debeaujon and Branchard, 1992) in 35mm Petri dishes in the dark, 27°C. After two weeks the protoplasts from each dish were subcultured into two dishes using either a pipette or forceps (if the cell clusters were adherent) and medium was diluted 1:1 with LCM2 medium (0.5 $mg.l^{-1}$ BA). The effect of handling the cells by forceps was checked after 2 weeks by comparing the viability of cells (stained by FDA). The dishes were kept in the dark, 27 °C and after two, three or four weeks were transferred into light (16 h day) conditions (23 °C) and the viability and number of cell divisions was checked after four weeks of protoplast culture. The cell clusters were then transferred onto solid F medium (Pelletier *et al*., 1983) in 6 cm Petri dishes. After four weeks microcalluses were put either on F medium or on MS medium (Murashige and Skoog, 1962). The size of microcalluses was measured at 12 weeks after protoplast isolation.

It was shown that using forceps for transferring cell clusters did not have negative impact on cell viability (Table 1).

Cells cultured for four weeks in the dark (27°C) had the highest viability and highest number of cell divisions (Table 2).

Microcalluses grown on MS medium were about 11% bigger then those grown on F medium (Table 3).

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Table 1. Effect of handling cell clusters with forceps

	Number of samples cultured	***Number of samples with living cells (4 weeks of culture)***
Cell clusters left in original dish	46	12
Cell clusters transferred by forceps into new dish	46	11

Table 2. Increasing viability and cell division number depending on length of the culture period in the dark at 27 °C

Length of period in the dark, 27°C	Protoplast viability after isolation*	Cell viability after 2 weeks*	Cell divisions after 2 weeks*	Cell viability after 4 weeks*	***Cell divisions after 4 weeks****
2 weeks				2%	1%
3 weeks				18%	6%
4 weeks				20%	8%
Average	70%	27%	2%	13%	5%

* *counted as percentage of all cells (including apparently dead ones)*
All percentages are derived from 12 samples (Petri dishes), for each sample average was counted from 10 readings.

Table 3. Size of microcalluses depending on solid culture medium type

	Culture medium	
Size (mm) of microcalluses after 12 weeks of cultivation	MS *(Murashige and Skoog, 1962)*	F *(Pelletier et al., 1983)*
Average	1,14	1,03
Standard error	0,04	0,03

Twelve samples (= Petri dishes) were measured for each medium. All microcalluses (35 in average) in each Petri dish were counted.

THE STUDY OF REGENERATION POTENTIAL *IN VITRO* OF *Vicia faba* L., *Lupinus albus* L. AND *L. Angustifolius* L.

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Reliable *in vitro* regeneration is a prerequisite for utilization of plant biotechnology in various crop improvement programmes. The regeneration protocols *in vitro* in faba bean and lupins were intensively studied during last two decades. Nevertheless, the availability of really reproducible protocols is still limited and restricted mostly on shoot organogenesis from pre-existing meristems (Tejklová *et al.,* 1984; Schulze *et al.,* 1985; Busse, 1986; Griga, 1988a,b; Selva *et al.,* 1989). *De novo* regeneration (organogenesis) from callus is strongly genotype-dependent (Galzy & Hamoui, 1981; Griga & Klenotičová, 1994; Tegeder *et al*., 1995) and protocols are rather complicated and sometimes difficult to reproduce. The only data on faba bean somatic embryogenesis were published by our laboratory (Griga *et al.,* 1987; Griga & Klenotičová, 1994). Similar situation is in *Lupinus* sp., where organogenesis from pre-existing meristems was the most common approach (Sator, 1985b, 1990; Sroga, 1987; Upadhyaya *et al*., 1992; Hardy *et al*., 1995) and only one paper reported somatic embryogenesis (Nadolska-Orczyk, 1992).

The objective of our research is to use a somaclonal variation phenomenon and *in vitro* selection methodology for improvement of faba bean and lupin resistance to fungal pathogens as an alternative approach to GMO technology. A partial objective is also to study an induction of pathogenesis-related proteins at tissue culture level. Here we present one-year data (2006) on optimization of regeneration protocols in selected faba bean and lupin cvs. which will serve as a background/experimental tool for above mentioned objectives.

Three cvs. of *Vicia faba* L. - Merkur, Merlin and Mistral, two cvs. of *Lupinus albus* – Amiga and Wat, and *L. angustifolius* cv. Boruta were included in the study; we used our previously developed faba bean or pea protocols modified with more recent literature data.

Multiple shoot organogenesis: In faba bean, three types of explants were used for multiple shoot induction. Shoot apices of 7-day-old faba bean seedlings were cultured on MSB-medium supplemented with 4.51 mg.l^{-1} BAP and 0.019 mg.l^{-1} NAA – B/O/1 (Griga, 1990). Embryonic axes from immature zygotic embryos (4-8 mm in length) were cultured on MSB-medium with 0.11 mg.l^{-1} TDZ and subsequently transferred to MSB-medium with 1.04 mg.l^{-1} GA_3 – B/O/2 (Sanago, 1996). Nodal segments from faba bean seedlings were cultured on MS-medium supplemented with 2.2 mg.l^{-1} TDZ, consecutively on MS-medium with 4 mg.l^{-1} TDZ and again on MS-medium with lower TDZ level (2.2 mg.l^{-1}) – B/O/3 (Tzitzikas *et al.,* 2004). In lupin, the nodal explants of 7 to 14-day-old seedlings and embryonic axes isolated from immature zygotic embryos were cultured either on MSB-medium with 4.51 mg.l^{-1} BAP and 0.019 mg.l^{-1} NAA or on MSB-medium supplemented with 5 mg.l^{-1} TDZ and then transferred onto MSB0-medium and MSB-medium with 4.51 mg.l^{-1} BAP and 0.019 mg.l^{-1} NAA (Fig. 1). Well growing shoots (10 mm in length) of both faba bean and lupins were rooted on rooting media (MSB-medium with 0.19 mg.l^{-1} NAA or 20 mg.l^{-1} IBA). The following parameters were recorded at the end of shoot organogenesis stage (2 months) and root induction stage (5 weeks): explant weight, number of explants forming shoots, number of shoots per explant, shoot length and number of rooted shoots.

Differences between single culture procedures of *V. faba* were observed (Fig. 2). In view of faba bean explant weight, the culture procedure B/O/2 (Sanago, 1996) was the most efficient, but in view of number of growing shoots per explant the procedure B/O/3 (Tzitzikas *et al.,* 2004) was more successful (Table 1). Regenerated faba bean shoots in these two variants did not produce any roots. The most advantageous procedure was B/O/1 (Griga, 1990) in the view of number of growing shoots per explant, shoot length as well as number of rooting shoots (15-45

% rooted shoots on MSB-medium with NAA and 5-20 % rooted shoots on MSB-medium with IBA – Table 1).

Somatic embryogenesis: The cotyledons of faba bean immature zygotic embryos were cultured on L2-medium (Phillips & Collins, 1979) with 0.55 $mg.l^{-1}$ 2,4-D and 25 $g.l^{-1}$ sucrose. In lupins, the shoot apices of 14-day-old seedlings and the cotyledons of immature zygotic embryos were placed on MSB-medium supplemented with 0.6 $mg.l^{-1}$ Picloram (Griga, 1998) or on B5-medium with 5 $mg.l^{-1}$ 2,4-D and 0.25 $mg.l^{-1}$ Kinetin and then transferred onto MSB-medium with 0.1 $mg.l^{-1}$ ABA and 0.1 $mg.l^{-1}$ Zeatin (Nadolska-Orczyk, 1992). The following parameters were recorded: callus morphology, callus colour, itensity of callus formation, number of explants forming somatic embryos, morphology of somatic embryos.

In faba bean, the used protocol resulted in non-embryogenic, light-green or yellow compact calli in all cultivars, somatic embryogenesis was not achieved. In lupins, embryogenic explants were achieved only in *L. albus* cv. Wat from cotyledons of immature zygotic embryos; however, globular somatic embryos did not develop into further developmental stages.

The regeneration protocols *in vitro* with the maximum yield of regenerated plants will be used in selection experiments. Well growing *Vicia* and *Lupinus* cultures will be subjected to culture filtrates of *Fusarium*, *Ascochyta* and *Colletotrichum* sp. to obtain somaclones with improved resistance to these pathogens. Future work will be also focused on plant-pathogen interaction and study of PR-proteins.

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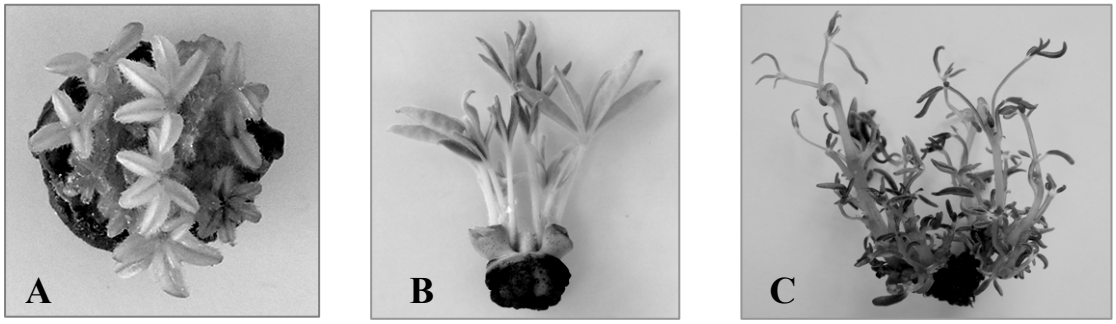

Figure 1 Multiple shoot formation in *Lupinus albus*, cvs. Amiga (**A**) and Wat (**B**) on MSB-medium with 4.51 mg.l^{-1} BAP and 0.019 mg.l^{-1} NAA and *L. angustifolius*, cv. Boruta (**C**) on MSB-medium with 5 mg.l^{-1} TDZ

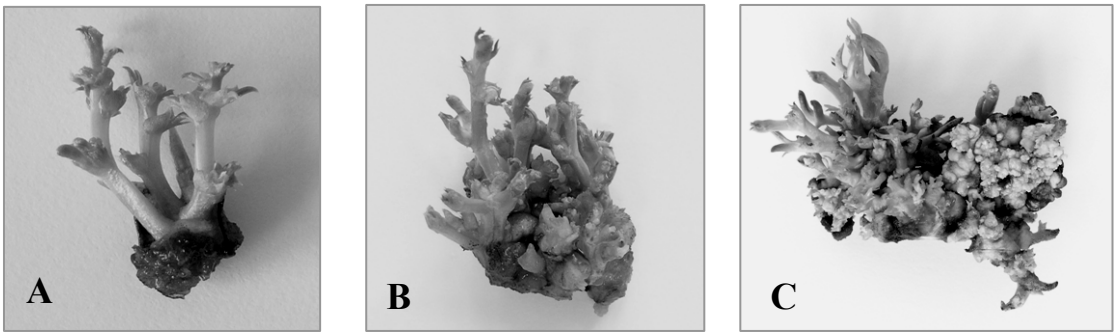

Figure 2 Multiple shoot formation in *Vicia faba* L., cv. Merlin: **A** – MSB-medium with 4.51 mg.l^{-1} BAP and 0.1 mg.l^{-1} NAA (B/O/1); **B** – MSB-medium with 0.11 mg.l^{-1} TDZ and consecutively MSB-medium with 1.04 mg.l^{-1} GA_3 (B/O/2); **C** - MS-medium supplemented with 2.2 mg.l^{-1} TDZ, consecutively with 4 mg.l^{-1} TDZ and again with 2.2 mg.l^{-1} TDZ (B/O/3)

Table 1 Morphological parameters of multiple shoot cultures in *Vicia faba* L. cvs. Merkur, Merlin and Mistral

PROCEDURE	REFERENCE	CULTIVAR	EXPLANTS FORMING SHOOTS [%]	EXPLANT WEIGHT [g]	GROWING SHOOTS PER EXPLANT	SHOOT LENGTH [mm]	ROOTED SHOOTS-NAA [%]	EFFICACY - NAA [%]	ROOTED SHOOTS-IBA [%]	EFFICACY - IBA [%]
B/O/1	Griga 1990	Merkur	94.57	0.8082±0.3254	3.83±2.76	26.99±8.27	35.23	**16.9**	22.5	**9.8**
		Merlin	86.96	1.2234±0,4112	5.20±2.55	20.21±5.53	45.45	**17.4**	5.95	**2.2**
		Mistral	33.46	0.7697±0.3713	3.26±1.98	16.53±6.49	15.63	**3.8**	4.69	**1.1**
B/O/2	Sanago *et al.* 1996	Merkur	7.41	1.1646±0.4956	0.90±1.29	4.65±6.19	x	x	x	x
		Merlin	15.87	1.4619±0.6027	3.15±2.46	10.83±6.02	20.0	**4.76**	2.78	**0.79**
		Mistral	14.81	1.5274±0.7025	2.19±2.02	8.10±6.63	25.0	**3.70**	0	**0**
B/O/3	Tzitzikas *et al.* 2004	Merkur	11.07	0.8318±0.4330	2.25±1.21	12.99±5.45	x	x	x	x
		Merlin	47.17	1.1816±0.2706	6.09±2.11	13.13±3.46	1.72	**0.94**	0.6	**0.94**
		Mistral	37.44	0.7845±0.3847	4.13±2.01	11.37±3.73	5.94	**2.53**	0.93	**0.42**

GALACTOGLUCOMANNANS ACCELERATE TRACHEARY ELEMENT DIFFERENTIATION IN *Zinnia* XYLOGENIC CELL CULTURE

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Oligosaccharides, including galactoglucomannan oligosaccharides (GGMOs) with anticipated signalling activity, play an important role in various aspects of plant growth and development. In the context of xylem differentiation, it is conceivable that GGMOs may be candidates that either act in directing cell fate determination or in more downstream events of differentiation.

We have compared global gene expression using *Zinnia* macroarrays (Pesquet *et al*., 2005) in the presence or absence of GGMOs in tracheary elements (TE)-inductive medium. These macroarrays contain protoxylem (PX) and metaxylem (MX) genes, since the library was made from cultures containing both types of TEs (Pesquet *et al*., 2003). Two comparisons were made: a pool of RNA (1) from cell cultured in the presence vs. absence of GGMOs for 12 h, and (2) from cells cultured in the presence/absence of GGMOs for 60 h. During the first 12 h GGMOs up-regulated genes characteristic for Stage 2, and down-regulated genes characteristic for Stage 1, suggesting an acceleration of the TE differentiation process. At 60 h, which corresponds to the onset of TE secondary wall deposition, the large majority of genes that showed modified expression were by GGMOs repressed rather than induced. The fact that the majority of genes were down-regulated may be explained by the fact that the macroarrays used were established with cDNAs from a first-burst PX-rich library. Since GGMOs support MX formation (results from physiological studies), it is reasonable to suppose that levels of PX-TE gene expression would decrease accordingly. From a structural and biochemical point of view also results that GGMOs affect secondary wall patterning. We compared differences in global gene expression in two experimental conditions that favour MX formation. One may predict that genes up-regulated in both comparisons may be associated with MX production whereas genes that are down-regulated in both comparisons would more likely be associated with the PX genetic program. Only less abundant genes were found in both comparisons. This set of genes includes the four cysteine proteinases, TED 4-3, and zIAA8. The overlapping of these genomic comparisons gives further weight to these down-regulated genes as potential actors in PX formation. These results highlight a novel physiological role for GGMOs in channelling TEs into a MX genetic program and provide a means to identify potential actors of the MX and PX pathways.

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IN VITRO POLLINATION IN THE GENUS *Cucumis*

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Cucumber (*Cucumis sativus*) belongs among 10 most important vegetables in world production. However, this crop is susceptible to many diseases and pests. Valuable sources of resistances to those have been found in wild African species of *Cucumis* (*C. melo* - line MR 1 has resistance to *Pseudoperonospora cubensis*). For incorporation of these resistance genes into cucumber it is necessary to use unconventional hybridization techniques, including various methods of *in vitro* cultures (Lebeda *et al*., 2007). The main reason of that is different chromosome number in *C. sativus* and wild *Cucumis* spp. (*C. sativus* belongs to an Asian group, n=7; *C. melo* and wild *Cucumis* spp. to an African group, n=12) (Jeffrey, 2001; Křístková *et al.*, 2003). One of the possible methods to overcome these crossing barriers in the genus *Cucumis* is *in vitro* pollination and fertilization (Ondřej *et al*., 2002).

Selected genotypes of *Cucumis* species were used for *in vitro* pollination experiments (*C. sativus* - CS, *C. sativus* – cultivated *in vitro* conditions - CSIV and *Cucumis melo* - CM). Immature female and male flowers were sterilized (1 min in 70% ethanol, 10 min in 2.5% chloramine, three times rinsed in sterile water in aseptic conditions) and they cultivated on ½ MS medium (Murashige and Skoog, 1962) in thermostat (25^{o} C) for 3 days. Then mature female flowers were excised in aseptic conditions and ovules were transferred onto solid media, which were tested (CP medium - Castano and De Proft, 2000; and YS medium – Ondřej *et al*., 2002). Pollen grains were isolated from anthers from mature male flowers by centrifugation in washing solution (modified NLN 13 medium, Lichter, 1981) and they were transferred on and around ovules on the culture media. The Petri dishes with ovules and pollen grains cultivated for 2 – 3 days in thermostat (25^{o} C) and then fertilized ovules were transferred onto 4 types of media supporting embryogenesis. The basic medium of these was MS medium and different types of tested media contained various additions as an ascorbic acid (OK medium), caseinhydrolysate (ON medium), coconut water (CW medium) and giberelic acid (GA medium) (Skálová *et al*., 2007).

The most important results were summarized in the Table 1. The YS medium was rather better for successful growth of pollen tubes and following fertilization (42%) than the CP medium (32%). On the other hand, the rising embryos cultured on CP medium was showed better consequent development after their transferred on media supporting embryogenesis (59%) than the embryos from YS medium (27%). The development of immature embryos on the media OK, ON, CW and GA was prosperous, the best results showed ON medium with addition of caseinhydrolysate (this supplement contained also the CP medium). Consequently, caseinhydrolysate could be evaluated as a suitable addition for development of early stage of embryos rising from *in vitro* pollination. From all types of *in vitro* pollination (summarized in the Table 1) was the pollination ♀ *C. sativus* × ♂ *C. sativus* (*in vitro* cultivated) the best resultful (29% obtained embryos) and than was the pollination of ♀ *C. sativus* × ♂ *C. melo* (18%). The most important factors for successful fertilization and following regeneration of embryos were the compositions of culture media and the maturity of ovules and pollen grains.

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Table 1. Number of isolated ♀ with ♂ around, of successful *in vitro* pollination and of regenerated E on two types of tested media (CP and YS)

♀	♂	No. of isolated ♀ (with ♂ around)		No. of successful fertilization		No. of regenerated E	
		CP	YS	CP	YS	CP	YS
CS	**CS**	660	660	150	190	15(OK) 45(ON) 35(CW) **95**	8(OK) 25(ON) 24(CW) **57**
CS	**CSIV**	140	140	60	110	24(OK) 15(ON) 9(GA) **48**	11(OK) 14(ON) 9(GA) **34**
CS	**CM**	160	160	100	100	25(ON) 9(CW) 7(GA) **41**	10(ON) - 7(GA) **17**
Total no.		**960**	**960**	**310**	**400**	**184**	**108**

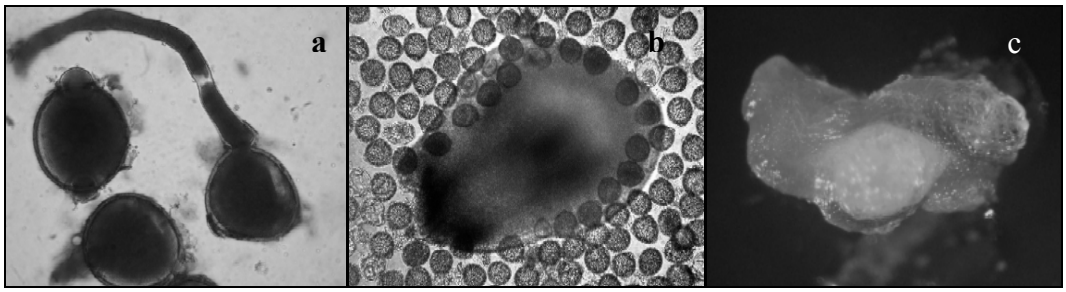

Figure 1. Mature pollen grains with pollen tubes (**a**); pollen grains around the isolated ovule (**b**); regenerated embryo (CS × CS, fertilized on CP medium, transferred on CW medium) (**c**)

A STUDY OF ASYMETRIC SOMATIC HYBRIDIZATION IN GENUS *Solanum*

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Optimalization of protoplast organelles inactivation (nuclei, chloroplasts, mitochondria) with goals using them in cybridization is aim of this study. The effort is focused especially on use extranuclei inheritance of wild species to increase variability of cultivated potato.

Inactivation of nuclei and semiautonomic organelles was studied in mesophyll protoplasts selected from the members of the genus *Solanum tuberosum* (cv. Kordoba, cv. Karin, cv. Komtesa) and *Solanum verrucosum*.

Immediately after the isolation, an aliquot of protoplasts was UV-irradiated (a germicidal lamp, 7 min and 12 cm), these protoplasts did not regenerate. Inactivation of semiautonomic organelles was performed in aliquots of protoplasts with graded concentrations of IODOACETAMIDE (0.3 mM; 0.4 mM), RHODAMINE 6G (0.05 mM; 0.1 mM) and IODOACETIC ACID (0.125 mM; 0.2 mM). These concentrations of active substances did not damage protoplasts and the regeneration of a cell wall was sporadically recorded. In controls, division was observed and microcolonies, microcalli and calli were derived. Protoplast fusions were realised by electric field and by PEG. So far, protoplasts were fused in combination: UV irradiated protoplasts + protoplasts treated with active substances (*S. verrucosum* UV + Kordoba IOAA 0.2 mM; *S. verrucosum* UV + Komtesa IOD 0.4 mM). Regeneration of these fused protoplasts and nonfused treated (UV or 0.2 mM IOAA or 0.4 mM IOD) protoplasts were evaluated. Fused protoplasts were more viable and regenerated cell walls in comparison with nonfused ones but higher stage of regeneration was not observed.

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CRYOPRESERVATION OF *Hypericum perforatum* L. SHOOT-TIPS BY VITRIFICATION METHOD

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Cryopreservation is a prospective method of the long term storage of plant material, as the original qualities of the treated plants are often preserved. It is based on the reduction and subsequent arrest of metabolic functions of biological material by imposition to ultra-low temperature. At the temperature of liquid nitrogen (-196 °C) almost all metabolic activities of cells are ceased. Therefore plant material can be preserved in this state for extended time periods.

In general, there are two types of cryopreservation techniques. Classical cryopreservation techniques involve freeze-induced dehydratation (as a result of slow cooling down to a defined prefreezing temperature, followed by rapid immersion in liquid nitrogen). Whereas in new vitrification-based procedures, concentrated cryoprotective media and/or air desiccation are used to dehydrate cells prior to freezing.

Only slow freezing method was applied for cryopreservation of *Hypericum perforatum* L. to date. Due to insufficient recovery rate of shoot tips cryopreserved by two-step cooling, the present study is aimed at application of the vitrification method, using several preculture agents and different length of preculture, on the shoot-tips of this species. Genetic stability of the recovered plants was determined at cytogenetic and molecular levels.

Shoot-tips of diploid *H. perforatum* plants derived from seeds of ten genotypes were subjected to cryopreservation using the vitrification method. The isolated shoot-tips with a couple of differentiated leaves were precultured in $RMB_{0.5}$ medium in the presence of 0.3 M sucrose for 16 hour, or in the presence of 0.076 μM ABA for 10 days, followed by treating of the shoot-tips with loading solution containing 2 M glycerol and 0.4 M sucrose, dehydration with plant vitrification solution (PVS2) and subsequent immersion in liquid nitrogen. Both, the length of preculture and type of preculture agents influenced the regeneration ability of meristems after cryopreservation. The survival rate - determined as percentage of meristems capable of differentiating plantlets - varied between 3.1 and 23.3 %. The highest value was achieved when the shoot-tips were precultured in the presence of 0.3 M sucrose for 16 hours. These results cannot be considered as satisfying and therefore the future experiments will deal with optimisation of the dehydration status of explants prior to cryopreservation.

Regeneration of shoots started after 3-4 weeks. The shoots regenerated after cryostorage did not show any morphological alterations as compared with the unfrozen controls. Genetic stability of the plants regenerated from cryopreserved meristems was eva- luated by means of flow-cytometry and PCR amplification of VNTR sequences and RAPD markers. The cytogenetic and molecular markers were compared in couples of treated and original control plant and are discussed in detail.

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EFFECT OF CULTIVATION ON CELL WALL MODIFICATIONS IN *Thlaspi spp*. ROOTS

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Thlaspi caerulescens is tolerates soils highly contaminated with zinc, cadmium and nickel and also can accumulate these metals to very high levels in the shoots. This species has been the subject of intense research to gain a better understanding of the mechanisms of heavy metal hyperaccumulation and tolerance and has been tested for its phytoremediation potential. Root structure of *T. caerulescens* is unusual by presence of lignified peri-endodermal layer, which is characterised by irregularly thickened inner tangential walls and in UV light it exhibits strong autofluorescence.

Thlaspi arvense is sensitive and non-accumulating species, closely related with *T. caerulescens*. This species is lacking the lingnification in peri-endodermal layer. These two contrasting species were grown in different conditions (filter paper and agar solified Murashige-Skoog medium) and development of their cell wall modifications in root was compared.

Seeds of *T. caerulescens* from metal polluted soil and seeds of *T. arvense* from non polluted habitat were sterilised (using detergent and aqueous solutions of NaClO and $HgCl_2$) and sown on medium in Petri dishes. For the first variant filter paper and distilled water were used. For the second variant Murashige-Skoog medium without vitamins and growth regulators was used. Seedlings were germinated and cultivated in growth chamber over a period of 6 days. Root samples were fixed and methanol.

For detection of suberin lamellae, free-hand sections of fixed roots were stained with Fluorol yellow 088. In *T. arvense* for Casparian band visualisation berberine-toluidine blue procedure was used. Visualization of Casparian band in *T. caerulescens* was possible only by TEM in ultrathin sections stained by $KMnO_4$.

Cell wall modifications of endodermis were in *T. caerulescens* formed closer to the root apex than in *T. arvense*.

In plants cultivated on filter paper thickening in peri-endodermal layer (present in *T. caerulescens*) starts at 3–4 mm from the root apex, Casparian band formation at 1–1.5 mm in *T. caerulescens* and at 3–4 mm in *T. arvense*. Suberine lamellae are formed at (5)–6–7–(8) mm in *T. caerulescens* and at 15–20 mm in *T. arvense*.

In plants cultivated on agar solified medium thickening in peri-endodermal layer starts at (1.5)–2–3–(4.5) mm from the root apex, Casparian bands formation at 0.5–1 mm in *T. caerulescens* and at 2–3 mm in *T. arvense*. Suberine lamellae are formed at (4)–5–7–(10) mm in *T. caerulescens* and at (5)–10–15 mm in *T. arvense*.

Observed interspecific differences may be connected with different tolerance of studied species. Differences between variants reflect the response of plant to different environmental conditions. Detail knowledge of cell wall modifications may contribute to better understanding of heavy metal accumulation and tolerance.

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ANTHER CULTURE IN BARLEY - STUDIES OF COLD PRE-TREATMENT

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In certain stress conditions microspores or young pollen grains can be switched from their normal pollen development towards an embryogenic pathway. This process is called androgenesis and represents an important tool for research in plant genetics and breeding. Androgenic embryos can germinate into completely homozygous, double haploid plants, which are very important for breeders and for genetic studies.

Androgenesis in anther cultures of spring barley (*Hordeum vulgare* L.) cultivar Amos has been investigated in our experiments. We focused our studies on biochemical analysis of anthers in different stages of cold pre-treatment (4 °C, 14 days) and during the first days of cultivation on induction media (MN6) (Chu and Hill, 1988).

Spectra of soluble proteins and superoxid dismutase (SOD) isozymes in anthers at different stages of cold pre-treatment and during first days of cultivation on induction media were compared and correlated with control. Number of anthers with induced microspores and number of structures originated from microspores were recorded, statistically evaluated. Results of biochemical analyses are discussed with structural observations. Androgenic induction was achieved with induction frequency (percentage of induced structures) 7.4 %. Five SOD isozymes were expressed in pretreated barley anthers. A new SOD isozyme (Rm 0.28) is expressed 2days after the transfer of anthers to induction media, and its activity continuously decreased. The analysis of soluble proteins spectra showed several qualitative (29 kDa, 53 kDa) and quantitative (59 kDa, 17 kDa) changes during cold pretreatment. The most substantial changes were found after 12 days of cold pre-treatment.

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LEVEL OF ENDOGENOUS GROWTH REGULATORS IN DIFFERENT HYPOCOTYL REGIONS OF FLAX CULTIVATED *IN VITRO*

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Flax (*Linum usitatissimum* L.) is an ancient cultivated species that still has an important impact on the world economy. This species is traditionally cultivated for its main products - fibre and seed oil (Preťová *et al*., 2005). Flax has been also the focus of a great deal of both basic and applied research in plant cell and biotechnology studies (Millam *et al*., 2005).

Morphogenic response of hypocotyl segments of flax cultivar Super were tested in liquid MS (Murashige and Skoog, 1962) media supplemented with 2,4-D (2 $mg.l^{-1}$ and 5 $mg.l^{-1}$). Hypocotyl segments, 2-3 mm in length, from apical, central or basal region of hypocotyl were cultured. Hypocotyl segments of flax cultivated *in vitro* six days on ½ MS medium with addition of 2 % sucrose and 0,8 % agar were used as control.

After two weeks of cultivation we observed that hypocotyl segments from apical and central part of hypocotyl regenerated better than hypocotyl segments from basal part. Following this observation, we measured the level of cytokinins in different regions of flax hypocotyl according to method (Dobrev and Kamínek, 2002). This method allows fast and efficient separation of cytokinins from auxin and abscisic acid. Our aim was to support hypothesis that endogenous growth regulators in plants influence the morphogenic response of explants.

We found out differences in content of cytokinins in apical, central and basal region of flax hypocotyl. The majority of determined cytokinins were located in hypocotyl segments from apical and central part of hypocotyl. Lower content of cytokinins were obtained from hypocotyl segments of control. The most abundant cytokinins found were the cytokinin ribosides.

The results suggest that the level of cytokinins differs in the apical and basal part of flax hypocotyl segments and may be also influenced by concentrations of 2,4-D added to MS media.

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Table 1. Level of cytokinins in apical, central and basal region of flax hypocotyl

Growth regulators	HF (control)			2 mg.l^{-1} 2,4-D			5 mg.l^{-1} 2,4-D		
Cytokinins (pmol.g^{-1})	A	C	B	A	C	B	A	C	B
Z7G	-	-	-	0,8	0,5	-	-	0,5	-
DHZ7G_1	-	-	0,4	0,3	-	-	-	-	-
DHZ7G_2	-	-	0,5	-	-	-	0,7	-	0,5
Z9G	-	-	-	1,2	-	-	0,7	0,2	0,1
ZOG	1,4	1,3	-	-	-	1,2	-	-	1,3
DHZ9G	-	0,3	-	0,2	0,3	-	-	-	-
DHZOG	-	0,6	-	3,5	3,9	1,0	1,3	0,7	-
Z	-	-	-	1,5	0,8	3,6	0,8	2,8	0,5
Z9ROG	-	0,4	-	17,0	23,0	20,0	6,8	10,0	0,6
DHZ	-	-	-	23,0	4,0	13,0	7,1	23,0	1,8
c-Z	2,2	1,4	0,8	0,9	0,7	-	1,4	0,8	1,0
DHZ9ROG	0,8	0,4	0,6	0,6	0,3	0,3	0,2	1,3	-
Z9R	3,0	1,5	1,3	5,7	0,2	0,1	4,1	17,0	0,4
IP7G	-	0,4	0,2	-	2,2	-	-	0,1	0,3
DHZ9R	3,4	0,7	0,8	28,0	-	0,3	0,9	21,0	-
c-Z9R	1,9	5,2	0,5	23,0	18,0	20,0	17,0	21,0	26,0
IP	0,3	0,4	0,5	0,7	-	-	0,6	0,2	-
IP9R	8,3	5,0	4,9	10,0	11,0	14,0	6,3	9,4	3,2

HF – hormone-free MS media
2,4-D – dichlorophenoxyacetic acid
A – apical part of hypocotyl
C – central part of hypocotyl
B – basal part of hypocotyls
Z7G – *trans*-zeatin-7-glucoside
DHZ7G_1 or **_2** – dihydrozeatin-7-glucoside (1, 2)
Z9G – *trans*-zeatin-9-glucoside
ZOG – *trans*-zeatin-O-glucoside
DHZ9G – dihydrozeatin-9-glucoside
DHZOG – dihydrozeatin-O-glucoside
Z – *trans*-zeatin
Z9ROG – *trans*-zeatin-9-riboside-O-glucoside
DHZ – dihydrozeatin, c-Z – *cis*-zeatin
DHZ9ROG – dihydrozeatin-9-riboside-O-glucoside
Z9R – *trans*-zeatin-9-riboside
IP7G – isopentenyladenine-7-glucoside
DHZ9R – dihydrozeatin-9-riboside
c-Z9R – *cis*-zeatin-9-riboside
IP – isopentenyladenine
IP9R – isopentenyladenine-9-riboside

SOMATIC EMBRYOS PRODUCTION IN LIQUID CULTURE OF *Narcissus* L. 'CARLTON'

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Protocols of *Narcissus* L. reproduction in liquid culture systems based on the process of somatic embryogenesis have not been elaborated up to the present. Previous works on *Narcissus* L. micropropagation in liquid media (Bergonon *et al*., 1992, 1996; Selles *et al*., 1997; Chen *et al*., 2001) showed that liquid culture systems were more efficient in comparison to cultivation on solid media. The use of liquid media may improve multiplication rates of *Narcissus* L. embryogenic callus and somatic embryos produced *in vitro*.

Embryogenic callus of *Narcissus* L. 'Carlton' generated from flower stem and ovary explants on MS medium (Murashige *et al*., 1962) with 3% sucrose, 0.7% agar and growth regulators: Picloram or 2,4-D (10 or 25 μM) and BA (1 or 5 μM) was cultured for 12 weeks in four systems: constantly on solid media, constantly in liquid media and cyclically in the 2-week or 4-week following cycles in liquid and on solid media. Additionally, for constant liquid media system the effect of the inoculum weight (0.5, 1.0, 1.5 g) on the embryogenic tissue growth was checked. The clusters of embryogenic callus obtained on ovary explants under the influence of 2,4-D (25 μM) and BA (5 μM) were cultured for 4 weeks in 100-ml Erlenmeyer flasks containing 25 ml medium consisting 25 μM of 2,4-D or 0.5 μM of NAA and 5 μM of BA. The cultures were maintained in the dark at 20 ºC. For liquid cultures a rotary-shaker at 100 rpm was used. Subcultures were carried out every 2 weeks. After 12 weeks in case of first experiment the number of somatic embryos and after 4 weeks in case of second experiment the number of somatic embryos and the rate of multiplication of callus were calculated.

The type of auxin and their concentration in a medium and the origin of embryogenic tissue were important factors in callus propagation and embryos formation. Callus derived on ovary explants had a higher regenerative potential in comparison to callus generated from flower stem explants. Picloram-treated embryogenic callus propagated better than induced with 2,4-D. However, callus cultivated in the presence of 2,4-D produced more somatic embryos. Picloram inhibited the process.

The ability to produce somatic embryos depended also on the type of culture system used for propagation. The highest number (21,1) of somatic embryos per 1g of inoculum tissue was noticed in case of embryogenic callus originated from ovary tissue and cultivated cyclically in the 4-week following cycles in liquid and on solid medium with 25 μM of 2,4-D and 5 μM of BA.

The density of inoculum influenced the multiplication of embryogenic tissue and somatic embryos formation. The highest biomass increase was observed on medium contained 0.5 μM of NAA and 5 μM of BA when the density of inoculum was 0.5g/25 ml of medium. When the density of inoculum was 1.5g/25 ml the highest number of somatic embryos was obtained.

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STRESS FACTORS: THE MECHANISMS CONTROLING DEVELOPMENT OF SOMATIC EMBRYOS

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Besides a potential for an inexpensive method of mass propagation, somatic embryogenesis (SE) of conifers and especially some spruces has been extensively utilized as a model system for investigating many aspects of a whole embryogenic process. Generation of embryos in culture through this process has become a model system for investigating factors affecting embryo's growth.

Conditions in *in vitro* tissue cultures expose explants to significant stresses as they are removed from their original tissue environment and placed on synthetic media. Main objective of this review is to draw attention to the most recent advances in research of SE of coniferous species, with emphasis on stages from embryo maturation to its germination.

Differentiated fate of plant cells, dependent on positional information and developmental signals, can be easily altered in *in vitro* conditions. Drastic changes in a cellular environment, such as exposing wounded cells or tissues to sub-optimal nutrient or *in vitro* conditions generate significant stress effects. Response to any stress conditions depends on two main aspects: level of the stress and physiological state of cells. If the stress level exceeds cellular tolerance, cells die. On the other hand lower level of stress enhances metabolism and induces adaptation mechanism. Adaptations include reprogramming of gene expression as well as changes in physiology and cell metabolism. Stress alters source regulation by switching on genes of specific enzymes in parallel with stress defense genes. Transient cell state induced by stress conditions can be characterized by extensive cellular reorganization and allows developmental switch, if appropriate signals are perceived.

Stress not only promotes dedifferentiation, but can be also used for induction of a somatic embryo formation. High salt concentration wounding, water stress, sugar treatment or osmotic stress positively influences somatic embryo induction in diverse plant species.

Conventional way to stress plant cells grown in *in vitro* conditions uses increased osmotic concentration of nutrient medium. Water stress of cultured cells can be imposed by elevated osmolarity of medium (using permeating or non-permeating osmotic) or by limiting the water availability from the cultured medium. Plasmolysing osmotic stress is exerted by sucrose and the non-plasmolysing stress by polyethylene glycol (PEG 4000). Consequently, non-plasmolysing effect of PEG is similar to the effect of water stress observed in cells of plants subjected to drought conditions (desiccation).

EFFECT OF UV RADIATION ON MESOPHYL PROTOPLASTS OF *Cucumis sativus*

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The possible application of UV irradiation for the donor cells in asymmetric hybridization was reported for *Beta vulgaris* (Hall *et al.*, 1992). Forsberg *et al.* (1998) applied UV irradiation as an alternative method for inactivation of nuclear components to create asymmetric hybrids between UV-treated *Arabidopsis thaliana* and *Brassica napus*. The aim of this work was to develop suitable and simple assays for the investigation of UV effect on mesophyl protoplasts of *Cucumis spp.* These assays are based on the spectrophotometric or fluorimetric measurements with microplate reader and will be used for study of cell physiology alterations in irradiated protoplasts during alternative method for asymmetric hybridization between *C. sativus* and *C. melo* or *C. sativus* and wild *Cucumis spp*. Cell vitality, cellulose biosynthesis, density of cell suspension and intenzity of 4´,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining were detected. Cellulose staining protocol with Calcofluore White (CW) was adapted for microplate reader (Glabraith, 1981). The influence of concentration of CW, signal dependence on incubation time and amount of cells were tested. CW in 0,001% final concentration and 10 min incubation time were selected for the next experiments. Negative effect of UV radiation on the cellulose biosynthesis is shown in Figure 1. Fluorescent signal of CW increased during regeneration of protoplasts only in control cells and this result was in agreement with histochemical observations. Nuclear DNA is very sensitive to UV radiation and expression of cellulose synthase, the key enzyme of cellulose biosynthesis, is strongly suppressed dependent on the intensity of UV irradiation (Perin, 2001). The vitality of protoplasts was assessed by fluorescein diacetate (FDA). Low vitality of irradiated cell, with tendency to further decreasing vitality, is shown in Figure 2. Both cellulose and vitality staining are suitable for monitoring of UV radiation effect on protoplasts. The third method used, measurement of the cell density, did not give significant evidence of UV influence on mesophyl cells (Fig. 3). Microscopy observation confirmed results from microplate reader measurements. DAPI staining was more suitable microscopic methods, because the of excitation and emission wavelength bandwidth of reader filters were too wide and changes in DAPI signal were not significantly different. The results for both tested genotypes of *C. sativus* were similar.

Materials and methods. Mesophyll protoplasts were isolated from 4-week-old *in vitro* cultured seedlings of two genotypes of *C. sativus* (09H390768, 09H390056). Protoplasts were isolated by enzymatic mixture of 1% Cellulase Onozuka R-10, 0.25% Macerozyme R-10 in Pgly solution (Gebeaujon and Branchard, 1992) and resuspended in LCM1culture medium (Debeaujon and Branchard, 1992) at the density of 10^5 protoplasts per ml. Immediately after isolation, a part of protoplasts was UV-irradiated (UV-C germicidal lamp, 10 min and 58 cm distance) in open Petri dishes and all dishes were cultured in a thermostat at 27 ^{o}C for 2 weeks in the dark. The detection of viability: fluorescent signal (λ_{exc} 485/20 nm, λ_{em} 516/20 nm) was measured after 5 min incubation in 100 µl of protoplast suspension (PS) after addition of 5 µl of fresh prepared 0,01% FDA (Pritchard, 1985). Cell wall cellulose level: 10 µl of 0,01% solution of CW was added to 100 µl of PS and after 10 min incubation was measured fluorescence signal (λ_{exc} 360/40 nm, λ_{em} 440/30 nm) (Galbraith, 1981). DAPI detection of dsDNA: 10 µl of 1000x diluted of stock solution of 0,01% DAPI was added to 100 µl of PS and after 5 min incubation was measured fluorescence signal (λ_{exc} 360/40 nm, λ_{em} 440/30 nm). Total cell density in PS was accurately estimated from measurement of optical density (OD 600 nm). The vitality, production of cellulose and DAPI signal were detected using fluorescent microscope Olympus BX-60 equipped with a DP70 digital camera. Isolation of protoplast and their UV radiation was done in

three independent experiments for both *C. sativus* genotypes. The experiments were repeated three times.

Acknowledgement

This work was supported by the Grant MSM 6198959215 from the Ministry of Education, Youth and Sports of the Czech Republic and by the project NAZV QF 4108.

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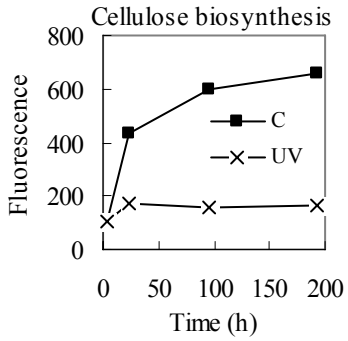

Figure 1. Cellulose biosynthesis during regeneration of mesophyll protoplasts from *C. sativus* (09H390768). Control cells ■, irradiated cells ×

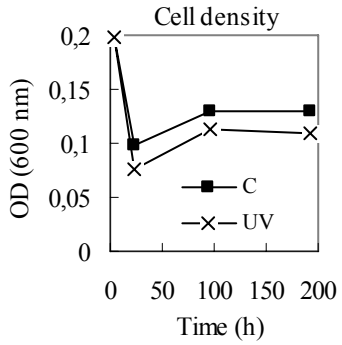

Figure 2. Cell vitality detected by FDA during regeneration of mesophyll protoplasts from *C. sativus* (09H390768). Control cells ■, irradiated cells ×

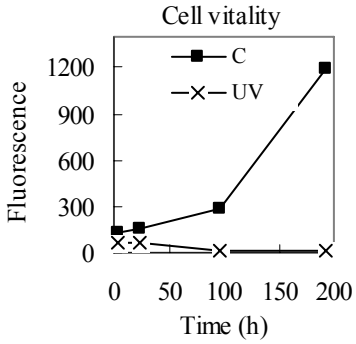

Figure 3. The density of protoplast suspension estimated from measurement of optical density (OD 600 nm). Control cells ■, irradiated cells ×

PLANTLET REGENERATION VIA SOMATIC EMBRYOGENESIS FROM OPEN-POLLINATED FAMILIES OF *Abies alba* TREES OF DOBROČ PRIMEVAL AND NURSE FOREST

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Somatic embryogenesis was initiated from immature zygotic embryos of *Abies alba* Mill. In 2006, one or two immature cones were collected in Jul 26th from open-pollinated (OP) families of 4 trees in Dobroč primeval and 3 trees in nurse forest. Cones were stored in paper pockets inside plastic bags at 4 ˚C for 3 days until immature seeds were dissected. Immature seeds were surface-sterilized for 10 min in 10 % H_2O_2.
Sixty to 132 explants were cultured per family, a total 500 megagametophytes inside with embryos were inoculated onto initiation SH (Schenk and Hildebrandt, 1972) medium with 1 $mg.l^{-1}$ 6-benzylaminopurine. Cultures were maintained at 24 ºC in the dark. Initiation of embryogenic suspensor mass (ESM) was evaluated every 7 days.

Maturation of somatic embryos was achieved culturing of ESM on modified MS medium (Murashige and Skoog, 1962) supplemented with 40 $g.l^{-1}$ maltose, 100 $g.l^{-1}$ polyethylene glycol-4000 and 10 $mg.l^{-1}$ abscisic acid. Five Petri dishes (60 x 15 mm) containing 300 mg of embryogenic tissue were used per treatment (per cell line). Cultures were maintained in the dark at 21-23°C for 8-10 weeks. Experiments were repeated twice.

Cotyledonary somatic embryos were subjected to a partial desiccation during three weeks at 24 ºC in the dark (Vooková et al., 1997/1998). Embryos were then transferred to germination medium containing ½-SH medium salts, 1% sucrose, 1% charcoal and 0.3% Phytagel

Embryogenic tissue was initiated on explants from 71.4 % of the studied families. Totaly five families responsed to initiation condition, three from four families (57 %) of the nurse forest and two from four families (50 %) of the primeval families. Initiation frequencies among OP families ranged from 4.5-56.2 % (nurse forest: 4.5-56.2 %, primeval: 5.4-16.8 %). Altogether, 64 embryogenic lines were established from 500 explants. However, great variation in the percentage of embryogenic line establishment was observed, depending on the OP family. Family of the nurse forest OL2 was superior to all the other families. Significant differences among other families of nurse or primeval forest were not observed

Of the 22 embryogenic cell lines established (two did not proliferated) from 2 primeval families and screened for somatic embryo maturation, 17 cell lines (77.3 %) showed maturation ability, 8 cell lines (36.4 %) produced cotyledonary somatic embryos. In nurse forest, 40 embryogenic lines representing 3 families was screened for somatic embryo maturation. Total, in 25 cell lines (62.5 %) embryo maturation was observed. Cotyledonary embryos developed only in 6 cell lines (15 %).

Mature cotyledonary embryos were converted into emblings and regenerants were obtained from 9 cell lines of Dobroč primeval and from 6 cell lines of nurse forest. Biochemically they were characterized by the variation in both soluble and insoluble protein profiles. The variation was characteristic for somatic embryos of individual cell lines rather than for the primeval and nurse stands. Within a group of 10 cell lines analyzed, the embryos of 3 lines possessed 60 kDa protein which was not detected in other

somatic embryos. Still other embryos derived from 2 cell lines lacked 20 kDa protein in their profiles. Small variation of insoluble protein profiles has also been observed among somatic embryos of individual cell lines at the levels of 70, 53 and 18 kDa proteins.

Enzymatically the embryos of 6 cell lines possessed 2 isoperoxidases as compared with 3-7 isoenzymes of the kind detected in the remaining somatic embryos. The 4 cell lines with enriched isoperoxidase pattern were characteristic by an increased number of their isoesterases as well. No indications were obtained supporting higher metabolic potential of somatic embryos derived from zygotic embryos of silver fir primeval stand as compared with its nurse stand.

Acknowledgement
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PLENARY SESSION II:
THE QUALITY CONTROL OF REGENERATED PLANTS

RETROTRANSPOSONS AS MAKERS AND MARKERS OF GENETIC DIVERSITY

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Transposable elements comprise the bulk of higher plants genomes, ranging from 15% of the nuclear DNA in *Arabidopsis thaliana* to more than 90% in some *Liliaceae*. The majority of these elements are the Class I LTR retrotransposons, which transpose *via* an RNA intermediate in a "Copy-and-Paste" mechanism. The process of retrotransposition is inherently error-prone and mutagenic, and closely resembles the life cycle of retroviruses. We are actively engaged in analyzing the various stages of that life cycle in barley, and in examining its control and induction mechanisms. Mobilization of retrotransposons gives rise to genetic and genomic diversity which not only is an important evolutionary process, but also can be used directly to track the changes that the retrotransposons themselves cause. Because retrotransposons represent a major share of the genome, make easily detectable genetic changes having known ancestral and derived states, and contain conserved regions for which PCR primers may be designed, retrotransposon insertions can be exploited as powerful molecular marker systems. Due both to their ubiquity and to the ease of isolating new elements in previously unstudied species, we have develop marker systems based on them for cultivated and wild plants including barley, wheat and wheat relatives, rye, oat, rape and turnip rape, oil palm, banana, and sunflower, tomato, grape, and blueberry to date. We develop and apply four key retrotransposon-based methods: SSAP, IRAP, REMAP, and RBIP. The SSAP, IRAP, and REMAP methods are multiplex and generate anonymous marker bands; RBIP scores individual loci, akin to microsatellite systems. The methods are variously suited to marker detection on agarose and polyacrylamide slab gels, slab and capillary sequencing devices, and arrays on solid supports. We apply them to marker-assisted breeding, phylogenetic analyses, biodiversity determinations, and evolutionary studies.

THE USE OF TY1-COPIA AND TRIM RETROTRANSPOSONS FOR FLAX GERMPLASM ANALYSIS

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In principle, three types of molecular polymorphism can be detected very simply by PCR, polymorphism of restricted fragments, insertional polymorphism and polymorphism in length between specific types of DNA sequences. Retrotransposons and microsatellites are some very interesting DNA sequences from the molecular genetic point of view. Both of them can be found as a part of intergene DNA and all the methods working with intergene DNA are seemed to be very suitable for searching wide genetics relationships expressing in the concrete genome.

Two LTR retrotransposons (Tst1 and FRODO) and a microsatellite sequence $(CAG)_3GC$ were chosen for primer designing and comparing for their ability to distinguish and find some phylogenetic relationships among a set of 36 flax (*Linum usitatissimum* L.)

Primers for these methods were designed in the case of Tst1 to match the 5´ end of 5´ LTR with the 5´end of 3´ LTR of another copy of this retrotransposon integrated in the DNA molecule. This primer is complementar to bases 1-28 and backward-facing (acession in NCBI database X52387). The another variant of the primer binding site are bases 4778-4800 and backward-facing (acession number in NCBI database X52387). Setting of FRODO retrotransposon, the primer was designed to match 3´end of 5´LTR with 3´end of 3´ LTR. The primer is corresponding to bases 1-28 and 597-615 and outward-facing (accession number in NCBI database AY860314).

Calculating out the primer differentiation ability index (Table 1) was based on the ability to distinguish first quartile of lowest 25% of possible unique fragments for interpretation of the effectivity of the primers to distinguish samples of whatever size.
Working with methods based on PCR, we work with primers derived from known LTR sequences. The primers can be complementary or corresponding to dsDNA. What is important is the fact of 5´LTR and 3´LTR congruity, thus, the PCR DNA fragments production can be realised using only the one primer. Designation of such primer must setting the strand polymerisation of the right orientation, according to the results we want to get.

Comparing the results of the used techniques can be seen the higher level of polymorphism for markers derived from Tst1 retrotransposon (76.9 %) as for FRODO retrotransposon (68,7 %). PCR-IRAP and PCR-RBIP analyses give us no evidential grouping based on straight pedigrees relationships, but the wider genetic backround of old land cultivars with modern ones can be seen. None of these populations has common characteristics of industrial type or pedigree, but the ties caused by evolutionary stress or old climatic and local effect causes retransposition is referred by retrotransposons and the ability of the flax genome to adapt to environment conditions is confirmed not only for retrotransposon markers.

PCR-REMAP reaction gives the highest level of polymorphism (86.4 %) but the ability to differ flax populations was lower then those of primer derived from Tst1

retrotransposon. In average, the polymorphism when the retrotransposon and microsatellite primers together in the PCR was about 20 % higher than using them in the PCR alone. It correspondences with the mechanisms creating the DNA molecule polymorphism for the retrotransposons and for the microsatellites distinctively. When it is analysed polymorphism of both of them, the retrotransposon activity, microsatellite mutations, recombinations and flax genome changes can express as higher level of polymorphism detected on the molecular level.

The use of effective techniques for germplasm characterization facilitates the conservation and utilization of plant genetic resources. Retrotransposons as molecular markers are high polymorphic and the active of them can distinguish even very similar genomes where other markes can fail. Those of retrotransposons no more active in plant genomes can conserve important marker site to analyse local or genetic development of the populations.

Acknowledgement

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Table 1. PCR profile characteristics of the used primers in different techniques

Primer (primer combination)	Total fragment levels	Percent Polymorphic	Total accession	Polymorphic fragment lebels	PIC[a]	Maximum possible unique fragments per technique	PDAI[b]
P-Tst1-01	13	76,9	36	10	0,420	9	0,176
P-Frodo2-02	16	68,7	36	11	0,542	9	0,215
P-Flax-2	20	65	36	13	0,594	9	0,211
P-Frodo2-02 + P-Flax-2	22	86,4	36	19	0,261	9	0,204

[a] *PIC = N (1 – $\sum P_i^2$) / (N – 1) where N is the sample size and P_i the frequency of the i-th pattern revealed by the technique summed across all patterns revealed by the technique*

[b] *PDAI = 0,25 – (the lowest number of unique fragments up to the maximum possible + the lowest number of unique absent fragments up to the maximum possible) / total sum of fragments*

GENERATION OF DROUGHT-RESISTANT TRANSGENIC CEREALS USING DREB FACTORS CONTROLLING STRESS TOLERANCE AT THE REPRODUCTIVE STAGE OF WHEAT DEVELOPMENT

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Transcription factors have been shown to control the activity of multiple stress response genes in a coordinated manner and therefore represent attractive targets for application in molecular plant breeding. Several families of transcription factors, such as DREB/CBF, ERF, MYK, MYB, AREB/ABF, NAC and HDZip class I and II, have been shown to be involved in the regulation of drought response in plants. The aim of our research is to investigate drought response at the reproductive stage of wheat development and the potential to increase drought tolerance during this stage. Using the yeast one-hybrid system and DRE as a bait (Lopato *et al.,* 2006) we have identified and cloned cDNAs of two novel DREB transcription factors from unstressed developing wheat grain that we expect to be involved in the initial drought response. These factors, designated as TaDREB2 and TaDREB3, were over-expressed in wheat and barley transgenic plants under constitutive (2X35S) and drought inducible (maize Rab17) promoters. Barley plants (cv. Golden Promise) were transformed using *Agrobacterium* mediated transformation; biolistic transformation was used to produce transgenic wheat (cv. Bobwhite). The presence of the transgenes in T_0 plants has been confirmed by PCR, Southern and Northern blot hybridization. T_1 transgenic plants with constitutive overexpression of *Ta*DREB2 demonstrated delay in germination and flowering; they were slower growing and darker than control plants. *Ta*DREB3 showed similar phenotype, except they were 1/3 smaller than wild type plants. T_1 transgenic plants demonstrated remarkable drought tolerance on the seedling stage of development. The drought tolerant phenotype correlated with the strong expression of transgenes. Detailed drought resistance analyses of transgenic cereals are in progress.

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THE DEVELOPMENT OF MOLECULAR MARKERS FOR RESISTANCE TO FIRE BLIGHT (*Erwinia amylovora*) IN APPLE AND PEAR

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Apples and pears are the most grown fruit species in Europe. In the last years, fire blight (*Erwinia amylovora*) has significantly begun to endanger the orchards. Fire blight has progressively spread on hawthorn from 1986 (Kůdela, 1990), when it was found in traditional fruit region around Litoměřice in 2000 and 2001. A use of resistant plant materials is one of the best possibilities how to defend from enhanced occurrence of this pathogen. Based on current knowledge, it was researched, that mechanism of resistance and architecture of individual plant resistance genes to different pathogens are very similar (Takken and Joosten, 2000). Plant resistance to different pathogens is mainly based on mechanism gene to gene. A specific resistance gene (R) recognizes a pathogenic virulence gene (Avr), wherewith starts a mechanism of hypersensitive response (HR) leading to systematic acquired resistance (SAR) and considerable reduction of infection (Takken and Joosten, 2000). Recently, it has been also researched, that majority of R genes contain exact conserve regions, multiple distributed in plant genomes. They are mainly nucleotide binding site (NBS) and leucine-rich repeat (LRR), also Toll/interleukin receptor (TIR) and leucine zip (LZ) in some cases. Within the NBS domain of resistance proteins there are several, small, presumably functional, conserved domains, such as the P-loop, kinase domains, MHD, and GLPL motifs (Meyers *et al*., 2003). These conserved motifs have been utilised in the design of PCR-based cloning and mapping strategies to characterise R genes. We have used these motifs directly for searching and detection of close sequences to fire blight resistance genes in apple and pear. In our experiment, we used 13 resistant, 12 sensitive and 8 middle genotypes of apples and 12 resistant, 10 sensitive and 5 middle genotypes of pears. Twenty eight PCR primers, derived from conserve regions of R genes, were used in direct PCR reactions or in combination with AFLP method (Hayes and Saghai Maroof, 2000), where DNAs were digested with restriction enzymes *Taq*I and *Apa*I. We found several polymorphic amplified fragments, which partly correlated with resistance to fire blight in apple and pears. (Figure 1) These fragments were cloned and sequenced and will be tested in specific PCR as molecular markers.

Acknowledgement

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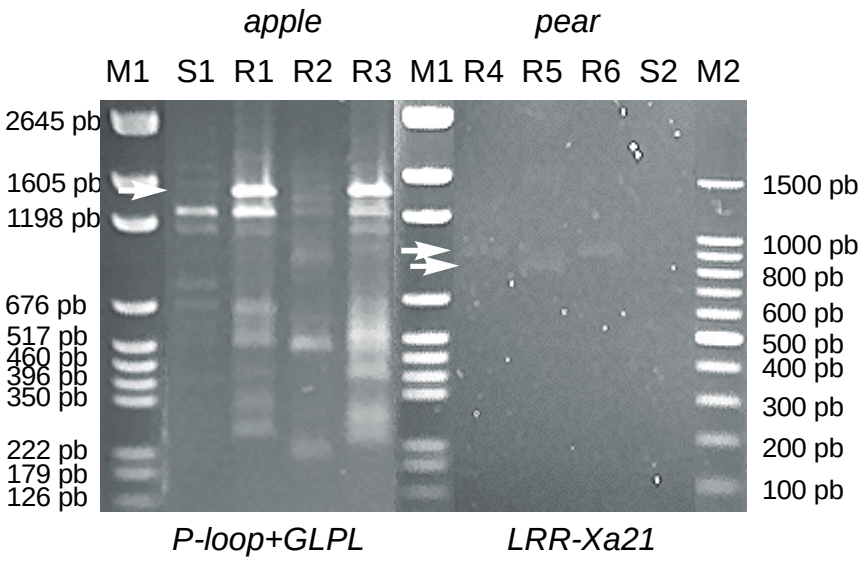

Figure 1. Analysis of amplified products in PCR reaction with primers derived from conserve regions of R genes in 1.5 % agarose gel for resistance and sensitive apple and pear genotypes. S1 – Gloster, R1 – Reanda, R2 – Close, R3 – Nova Easygro, R4 – Harrow delight, R5 – *Pyrus ussuriensis*, R6 – US 625-63-4, S2 – Boscoop, M1 – pGEM DNA marker, M2 – 100 bp ladder (Promega, Madison, WI, USA).

DEVELOPMENT OF DIAGNOSTIC MOLECULAR MARKERS FOR *Salvia spp*.

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The genus *Salvia* contains about 1000 species and more than 40 species of the genus are endemics in the Mediterranean region. The species of *Salvia* offers commercially important phytochemical properties for medicine and food industry. However, identification of the genotypes in the genus is complicated due to the morphologic similarity within the genus and/or the common occurrence of natural hybridizations.

This study utilized directed amplification of minisatellite DNA polymerase chain reaction (DAMD-PCR) to determine the possibilities of the development of species/genotype specific DAMD-PCR markers that would enhance species identification in the genus. Plant materials were collected from five natural populations growing in the Mediterranean Basin of Turkey. Two to 3 g of leaf samples were collected from each population and brought to laboratory, powdered using liquid nitrogen. DNA extraction, quality and quantity of the extracted DNA were performed according to Karaca *et al*., (2005).

Determination of species specific DAMD-PCR markers in this study was based on the following criteria: a species specific marker was defined as a distinct and reproducible DNA fragment presented in one species but it was absent in the rest of the species. PCRs and agarose gel electrophoresis of those species specific DNA markers were repeated twice for confirmation. Unweighted pair group mean average (UPGMA) method based on the Jaccard's coefficient was implemented using the software MVSP version 3.13O. Using a total of 12 universal minisatellite primers (Kang *et al.*, 2002) we determined a total of 14 DNA markers; one specific to *Salvia dichroantha Stapf.*, three specific to *Salvia fruticosa* Mill., five specific to *Salvia sclarea* L., one specific *Salvia tomentosa* Mill., and four specific *Salvia virgata* Jacq. We listed the diagnostic DAMD-PCR markers with approximate sizes in base pairs and relevant information in Table 1. Species diagnostic marker sizes range from 340 to 3000 base pair long.

Our study also clearly indicated that species in *Salvia* have a wide genetic diversity. The mean genetic similarity among the five species was 0.271 (±0.171) and ranged from 0.134 (between *Salvia sclarea* L. and *Salvia sclarea* L.) to 0.610 (*Salvia tomentosa* Mill. and *Salvia dichroantha* Stapf.). These diagnostic DNA markers would enhance the identification of an unknown sample from the other samples in a given experimental procedure. Therefore, these DNA markers are extremely important in germplasm characterization, conservation and utilization of plant genetic resources in the genus *Salvia* since many morphological traits used in the identification of *Salvia* are limited, difficult to score and under the influence of environmental factors.

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Table 1. Primers and species used, and determined diagnostic DAMD-PCR markers

Primer	Primer sequence 5'→3'	*Salvia* Species	Marker (bp*)
URP13R	TACATCGCAAGTGACACAGG	*Salvia dichroantha* Stapf.	3000
URP2F	GTGTGCGATCAGTTGCTGGG	*Salvia fruticosa* Mill.	480
URP1F	ATCCAAGGTCCGAGACAACC	*Salvia fruticosa* Mill.	2700
URP9F	ATGTGTGCGATCAGTTGCTG	*Salvia fruticosa* Mill.	2600
URP1F	ATCCAAGGTCCGAGACAACC	*Salvia sclarea* L.	390
URP2F	GTGTGCGATCAGTTGCTGGG	*Salvia sclarea* L.	2600
URP2R	CCCAGCAACTGATCGCACAC	*Salvia sclarea* L.	340
URP4R	AGGACTCGATAACAGGCTCC	*Salvia sclarea* L.	740
URP30F	GGACAAGAAGAGGATGTGGA	*Salvia sclarea* L.	740
URP13R	TACATCGCAAGTGACACAGG	*Salvia tomentosa* Mill.	2000
URP2F	GTGTGCGATCAGTTGCTGGG	*Salvia virgata* Jacq.	1000
URP17R	AATGTGGGCAAGCTGGTGGT	*Salvia virgata* Jacq.	860
URP17R	AATGTGGGCAAGCTGGTGGT	*Salvia virgata* Jacq.	980
URP25F	GATGTGTTCTTGGAGCCTGT	*Salvia virgata* Jacq.	750

** Sizes of the DAMD-PCR markers are given in approximate (bp)*

Figure 1. An UPGMA tree of 5 *Salvia* species (left) and .DAMD-PCR amplicons. The tree was generated using the Jaccard's similarity matrix based on the DAMD-PCR markers

Table 2. Similarity matrix of 5 *Salvia* species based on the DAMD-PCR markers and Jaccard's coefficient

		1	2	3	4	5
1	*Salvia sclarea* L.	1.00				
2	*Salvia fruticosa* Mill.	0.157	1.00			
3	*Salvia tomentosa* Mill.	0.190	0.464	1.00		
4	*Salvia virgata* Jacq.	0.134	0.216	0.213	1.00	
5	*Salvia dichroantha* Stapf.	0.135	0.453	0.610	0.137	1.00

PCR BASED DETECTION OF GMO POTATOES

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Genetically modified (GM) or transgenic crops, now more often called ***"Biotech crops"***, they are commercially cultivated since 1996. And also since 1996, the first year of commercialization of biotech crops, GM potatoes were cultivated in USA, Mexico, Canada and later in South Africa, China and India. Despite the continuing debate on biotech crops, particularly in countries of the EU, large and small farmers in industrial and developing countries continue to increase their plantings of biotech crops. The global area of approved biotech crops in 2006 was 102 million hectares. Remarkably, the global biotech crop area increased more than sixty-fold in the first eleven years of commercialization, making them the fastest adopted crop technology in recent history. The rapid adoption and the commercialization of biotech crops, during the initial ten-year period 1996 to 2005, reflects the substantial multiple benefits. Beyond the traditional agricultural products of food, feed and fiber, entirely novel agriculture products will emerge pharmaceutical products including oral vaccines, special and fine chemicals. The other use of biotech crop resources can be seen in replacing non-renewable, polluting, and increasingly expensive fossil fuels.

In 2006, 22 countries grew biotech crops, 11 developing countries and 11 industrial countries. The Czech Republic is on of the six EU countries where biotech crops are cultivated at present. The most compelling case for biotechnology, and more specifically biotech crops, is their capability to contribute to: increasing crop productivity and stability of productivity and production; conserving biodiversity, as a land-saving technology; the production of renewable resource based bio-fuels.

Commercially available biotech potato cultivars are improved with regard to resistance to potato leaf roll virus (PLRV), resistance to late blight (LBR), and insects (Bt Potato). In the nineties transgenic potatoes were the fifth more cultivated biotech crop. Although at present a range of other crops is more important, biotech potatoes are cultivated in many countries and has a great potential - for food and non-food purposes.

This study was focused on developing of fast, precise and cheap method based on PCR to detect the presence of transgenes in potatoes - tubers and leaves, allow monitoring the presence of GM potatoes in market, environment, etc. and to quantify "contamination" of ware potatoes (tubers) with GM ones.

Plant material. Transgenic cultivar Desiree-GM/GNA and non-transgenic Desiree as control were analyzed. GM potatoes were obtained from Potato Research Institute in H. Brod, non-transgenic tubers were obtained from CISTA Lípa u H. Brodu.

Extraction method. DNA was isolated from 100 mg of tubers or leaves using modified CTAB-PVP procedure according to Williams and Rogers. DNA was dissolved in 100 µl of sterile water and stored in -20˚ C.

PCR analysis. Methods for detection of lectin transgene by multiplex PCR and quantification of GM potatoes in mixture of transgenic and non-transgenic tubers by standard multiplex PCR and qPCR were developed.

Acknowledgement
This study was supported by grants MSM 60076658-06, NAZV 1B44011, IG 05/07.

DIGITAL DIFFERENTIAL DISPLAY TOOLS FOR MINING MICROSATELLITE CONTAINING ORGANISM, ORGAN AND TISSUE

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Tandemly repeated DNA sequences known as tandem repeats (TRs) are one of the most interesting features of every genome analyzed so far. A large number of computational methods and the software for the detection of TRs have been developed during the last decades. Among the TRs, microsatellites or simple sequence repeats (SSRs) have attracted many researchers since their significant presence in the Expressed Sequence Tags (ESTs) or in the cDNA libraries. With the advent of new biotechnological tools number of ESTs deposited in GenBanks is rapidly proliferating. There is a need for informative bioinformatics tools to assist the analysis and interpretation of these DNA sequences for microsatellites. We have developed two utility tools; Organism Miner (OrgMiner) to collect and sort organisms, and Keyword Finder (KeyWordFinder) to extract organ, tissue and development stage specific EST databases. OrgMiner utility sorts and splice GenBank and EST formatted DNA data files according to organism list requested by the user. KeyWordFinder extracts and collects keywords such as organs, tissues, cell lines or development stages for organism(s). We demonstrated applications of these utilities on *Gossypium* and *Capsicum* ESTs and observed microsatellites within the same organisms (*Gossypium* or *Capsicum*) show organ or tissue specify and differential expression in stage of development. Software reported in the present study will help in developments of microsatellite primer pairs for amplification of organ, tissue and development stage specific microsatellites. OrgMiner and KeyWordFinder utility tools were written in C++ using Microsoft Visual C++ software and currently run on Windows 98, Windows NT, Windows ME and Windows XP. The programs, and the sample data sets are self-extracting files and freely available from ftp://ftp.akdeniz.edu.tr/Araclar/TRA/ or can be obtained upon request from the corresponding author.

THE POSITIONAL CLONING AND CHARACTERIZATION OF THE *SCABRA3* GENE IN *Arabidopsis thaliana*

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In many plant species, a subset of the genes of the chloroplast genome is transcribed by RpoTp, a nuclear-encoded plastid-targeted RNA polymerase. We describe the positional cloning of the *SCABRA3* (*SCA3*) gene, which was found to encode RpoTp in *Arabidopsis thaliana*. We studied one weak (*sca3-1*) and two strong (*sca3-2* and *sca3-3*) alleles of the *SCA3* gene, the latter two showing severely impaired plant growth and reduced pigmentation of the cotyledons, leaves, stem and sepals, all of which were pale green. The leaf surface was extremely crumpled in the *sca3* mutants, although epidermal cell size and morphology were not perturbed, whereas the mesophyll cells were less densely packed and more irregular in shape than in the wild type. A significant reduction in the size, morphology and number of chloroplasts was observed in homozygous *sca3-2* individuals, whose photoautotrophic growth was consequently perturbed. Microarray analysis showed that several hundred nuclear genes were differentially expressed in *sca3-2* and the wild type, about a quarter of which encoded chloroplast-targeted proteins. Quantitative RT-PCR analyses showed that the *sca3-2* mutation alters the expression of the *rpoB*, *rpoC1*, *clpP* and *accD* plastid genes and the *SCA3* paralogs *RpoTm* and *RpoTmp,* which respectively encode nuclear-encoded mitochondrion- or dually-targeted RNA polymerases. Double mutant analysis indicated that *RpoTmp* and *SCA3* play redundant functions in plant development. Our findings support a role for plastids in leaf morphogenesis, and indicate that RpoTp is required for mesophyll cell proliferation.

Acknowledgement

This research was supported by the Marie Curie grant of the European Commision Human Potential Programme, Research Training Network (HPRN-CT-2002-00267).

EXPRESSION OF THE JAPANESE QUAIL GENE, ENCODING A HIGH METHIONINE-CONTAINING PROTEIN, IN TRANSGENIC PLANTS OF ALFALFA (Medicago sativa L.)

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Alfalfa (*Medicago sativa* L.) is an important forage crop and a source of protein in animal feed. However, like many other legumes, it is deficient in the essential amino acids (EAAs) methionine (Met) and cysteine (Cys). In alfalfa, the S-containing amino acids each constitute only about 1% or less of crude proteins (Frame *et al*., 1998), which is significantly less then the 3.5% Met+Cys content in a FAO reference protein (FAO, 1973). Recent advances in biotechnology allow the use of the transgenic approach to increase the content of specific essential amino acid in target plants. Several different molecular approaches have been developed to address this issue, including over-expression of a heterologous or homologous Met-rich protein, expression of a synthetic protein, modification of protein sequence, and metabolic engineering of the free amino acid pool and protein sink (Sun & Liu, 2004).

Up to now, a number of genes coding for high-Met proteins have been isolated and cloned. Among them, the most frequetly used for genetic transformation of different plant species have been the genes *Bn*2S (2S albumine from Brazil nut), *sfa*8 (sunflover seed albumine 8) and maize β-zein gene. We used a transgenic approach to introduce a cDNA of Japanese quail (*Coturnix coturnix*) gene *Ov* into the genome of alfalfa. The *Ov* gene codes for an ovalbumin protein of 383 amino acids from the oviduct of the bird. It contains highly balanced amino acid composition with 5.7% content of Met+Cys (Mucha *et al*., 1991).

Agrobacterium tumefaciens-mediated genetic transformation was used to introduce the T-DNA of plasmid pPDE1001Ov into alfalfa. The plasmid contained the cDNA of *Ov* gene uder the control of *CaMV* 35S promoter and *nos* terminator, and the *npt*II gene under the control of *nos* promoter and *ocs* terminator. Seven hundred and fifty four explants derived from petioles and leaflets of *in vitro*-grown plantlets of the highly embryogenic genotypes Rg9/I-14-22 and Rg11/I-10-68 of alfalfa (Faragó *et al*., 1997) were co-cultured with cells of *A.tumefaciens*. Selection of transgenic calli and regenerants was accomplished on selection media containing 50 $mg.l^{-1}$ kanamycin (Kn). In total 105 Kn^R regenerants, 90 shoots from Rg9/I-14-22 and 15 shoots from Rg11/I-10-68, were obtained 8 months after the inoculation of explants. The shoot cultures were maintained for another 6 weeks on a rooting medium supplemented with a double-concentration of Kn, where only the putatively transgenic plants could form roots.

Biological (Kn rooting assay, paromomycin leaf bleach assay), molecular (PCR, Western blotting) and biochemical analyses (HPLC) were performed to characterize the putatively transgenic plants. Control, non-transgenic isogenic lines, were not table to root (0 of 24 plants) on 100 $mg.l^{-1}$ Kn containing media, while the rooting percentage of putatively transgenic lines ranged from 25.0 – 87.5% (in average 54.7%). The paromomycin leaf bleach assay showed a significantly higher Kn resistance level (I_R = 2.50 – 6.25, 1-7 scale) in putatively transgenic plants than in non-transgenic control (I_R = 2.0).

PCR analysis showed the presence of 496 bp fragment of *Ov* gene in 96.3% (26 of 27 plants analyzed) putatively transgenic plants. Twenty seven transgenic plants were also analysed using Western blotting for the expression of the introduced *Ov* gene. Accumulation of ≈ 43 kDa transcript was detected in leaf samples of 8 lines. The concentration of ovalbumin in leaves of *Ov*-positive plants was estimated to be in the range of 0.01 – 0.1% TSP.

We used also HPLC for analysis of amino acid composition of bulked leaf+stem samples of 32 transgenic and 3 non-transgenic control lines of alfalfa. Of these, three lines (Figure 1) con-

tained 188-223% Met+Cys comparing to non-transformed control (0.228 % DW). However, there were also transgenic lines with lower than control-level Met+Cys content.

In conclusion, we used a transgenic approach of introduction of high-Met containing heterologous protein gene into the alfalfa genome in an attempt to increase the EAA content in this crop. The putatively transgenic plants showed the presence of introduced *Ov* gene sequences, as well as the accumulation of the respektive transcript. However, the detection of ovalbumin protein in only 30% transgenic plants and the wide range of Met+Cys content in these plants suggest a role of ovalbumin instability, position effect and/or somaclonal variation in transgenic plants. Further study is needed to elucidate the fate of ovalbumin in our transgenic alfalfa plants.

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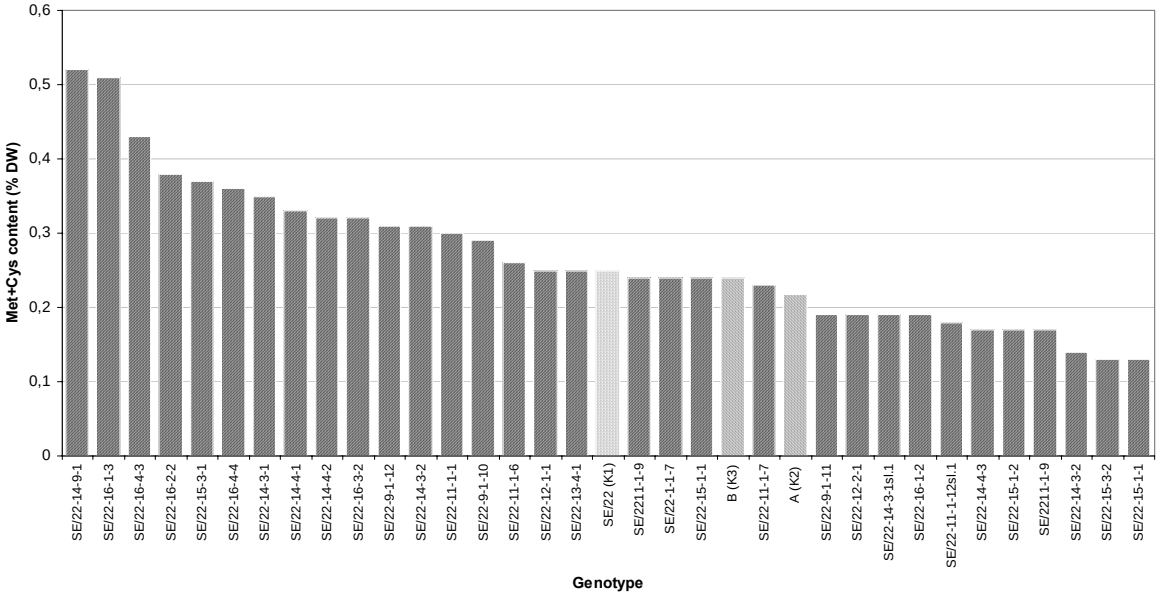

Figure 1. Protein-bound amino acid analysis in 32 transgenic and 3 non-transgenic (control, K1 = non-transgenic Rg9/I-14-22; K2 and K3 = non-transgenic seed-derived plants of cultivar Lucia) lines of alfalfa

Posters

IMPROVEMENT OF WHEAT TECHNOLOGICAL QUALITY BY MOLECULAR MARKERS

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Glutenins are a major component of the storage proteins in wheat (*Triticum aestivum* L.). Of these, high molecular weight (HMW) glutenin subunits are the key factors that influence bread-making quality, dough strength and elasticity. Specific types of HMW glutenin subunits are categorized according to mobility in the SDS-PAGE which is routinely used method at the protein level. The SDS-PAGE method has also its limitations. Apart from, the analyses can be carried out only from seeds, SDS gels are not well suited for automated high-throughput allele genotyping due to the need of manual interventions. The application of DNA markers for discriminating HMW glutenin genes offers several advantages over SDS-PAGE. They are detectable at all stages of plant growth and are not affected by the environment. PCR-based molecular markers provide a powerful tool for high-throughput and cost-efficient genotyping due to their high potential for automation in the multiplexed PCR assay.

The novel HMW glutenin subunits associated with a good dough quality of wheat were identified in the set of analysed wheat cultivars by SDS-PAGE. Thus, these HMW glutenin subunits could be valuable for the improvement of bread-making quality in wheat breeding, in addition to increase genetic diversity of the common wheat.

The aim of the work was to analyze 25 wheat genotypes (*Triticum aestivum* L.) on the base of the proteins and DNA molecular markers. Storage proteins were separated by SDS-PAGE and A-PAGE respectively, recommended by the international organization ISTA. Results showed, that electrophoretical profile HMW-GS with composition 0, 7+9, 5+10 by SDS-PAGE was the dominating one. There was also used separation of gliadins by A-PAGE for detection of secalin block, which was observed in 9 genotypes. DNA markers have now higher application in the detection of genes controlling important wheat properties. There were analyzed the same set of wheat genotypes on DNA markers. Wheat cultivars were analyzed for the presence of major HMW alleles at A, B and D genome loci with multiplexed PCR assay and then were compared with protein analysis done by SDS-PAGE. PCR analysis for gene controlling bread-making quality confirmed that used primers can be utilized for detection of wheat technological quality.

Acknowledgement
This work was supported by a VEGA project No. 1/3474/06.

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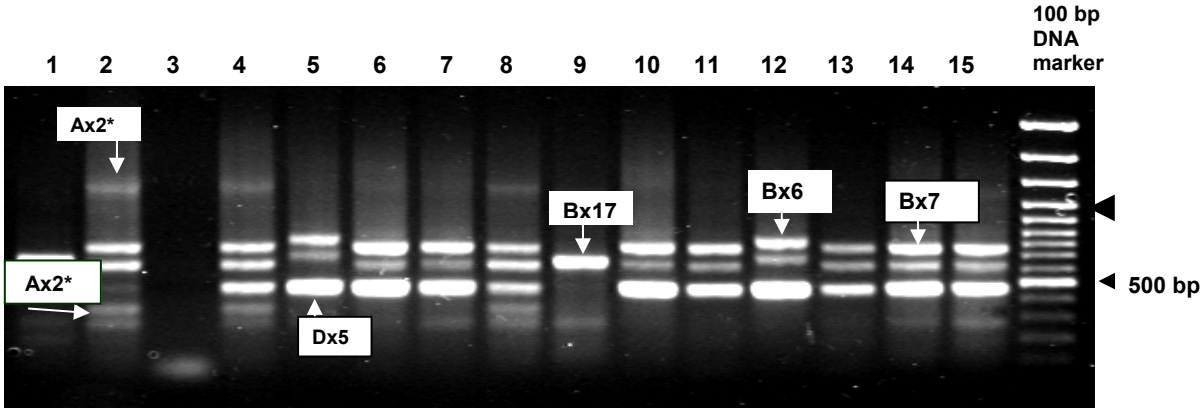

Figure 1. Multiplex PCR for detection of HMW-GS genes: 1 Marquis, 2 Velta, 3 Arida,4 Istar, 5 Armelis, 6 SO-296, 7 SO-355, 8 SO-496, 9 Vanda, 10 Petrana, 11 SK-22, 12 SK-26, 13 SK-30, 14 MV-06-95, 15 MV-230-96

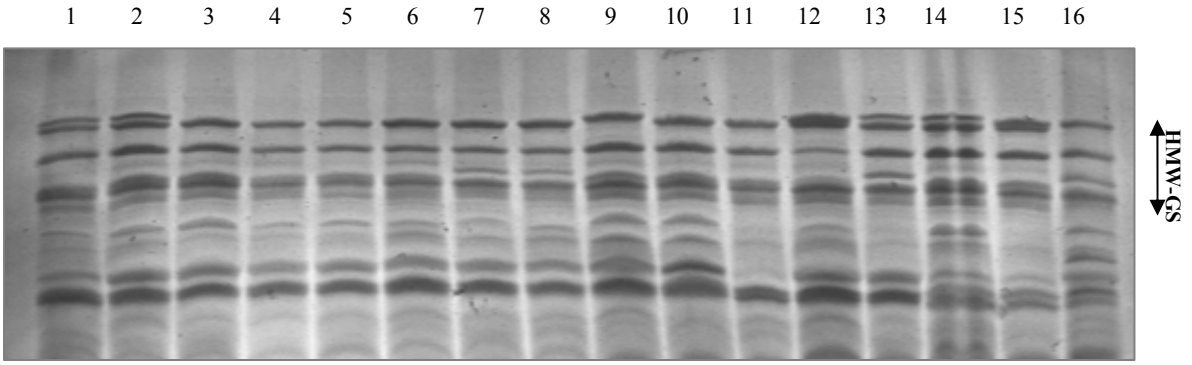

Figure 2. Electrophoretical profile HMW-GS in *Triticum aestivum* L.: 1 Marquis, 2 Velta, 3 Arida,4 Istar, 5 Armelis, 6 SO-296, 7 SO-355, 8 SO-496, 9 Vanda, 10 Petrana, 11 SK-22, 12 SK-26, 13 SK-30, 14 MV-06-95, 15 MV-230-96, 16 Chinese Spring

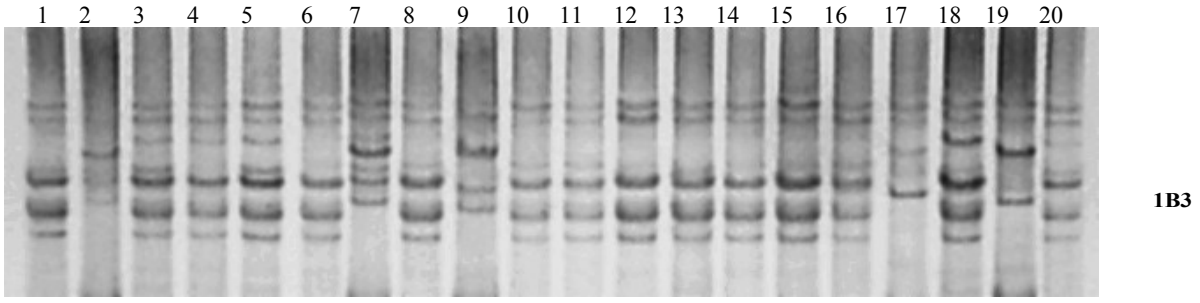

Figure 3. Electrophoretical spectrum of gliadins in A-PAGE.

1 Marquis, 2 Velta, 3 Arida,4 Istar, 5 Armelis, 6 SO-296, 7 SO-355, 8 SO-496, 9 Vanda, 10 Petrana, 11 SK-22, 12 SK-26, 13 SK-30, 14 MV-06-95, 15 MV-230-96, 16 Chinese Spring, 17 Astella, 18 Ilona, 19 Brea, 20 Torysa

COMPARATIVE ANALYSES OF TRANSCRIBED AND UNTRANSCRIBED MICROSATELLITES

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The existence of simple sequence repeats (SSRs) or microsatellite DNA in expressed sequence tags (ESTs) or cDNAs has attracted many researchers for developing EST-SSR markers in numerous plant and animal species. The development of EST-SSR primer pairs is very convenient and economic. However the polymorphism information content (PIC) of the EST-SSR (transcribed) is usually low in comparison to conventional SSR (untranscribed) primer pairs. In the present study we investigated several criteria that seem to affect the PIC. Criteria included i) the type of SSRs; simple (SSR) and complex (CSSR) and compound (CSR) sequence repeat, ii) the number of repeats (ranged from 3 to 40 nucleotides in length), iv) the motif type (mono-nucleotide to hexa-nucleotide) and v) the source of EST (three species in cotton and 2 species in pepper). We also compared phylogenic relationship among several *Gossypium* species using the EST-SSR and conventional SSR primer pairs.

We developed a total of 500 EST-derived microsatellite primer pairs using *Gossypium spp*. (cotton) and *Capsicum spp*. (pepper) expressed sequence tag (EST) in the EST database (dbEST, GenBank). Selected EST-SSR primer pairs and conventional SSR primer pairs were commercially synthesized and used on lines, cultivars, accession, species and F_2 populations of cotton and pepper.

Results of the present study indicated that polymorphism information content (PIC) increased as the number of repeat in a SSR increased (Figure 1). The minimum number of SSR should be greater than 8 for higher level of polymorphism (Figure 2). Type of motif is also found to be related with the higher level of PIC. Repeat motifs of AG, CT, GA, GAA and TC showed higher level of PIC (Figure 3). The level of PIC for GT motif was the lowest statistically among the motif types investigated. Study also revealed that the type of SSR showed different levels of PIC values (Figure 4). Among the type of SSR the highest PIC value was observed in complex SSR. The lowest PIC value was observed in simple SSR. Our results also indicated that there was no association between higher values of PIC and mono-, di-, tri- and tetra-nucleotide repeat. The use of different sources (species and genus) clearly indicated that transferability of EST-SSR among the species were higher than between the genus. Comparison of the phylogenies of genomic (untranscribed) and EST-SSR (transcribed) showed the same relationships indicating that EST-SSR primer pairs are equally valued as the genomic ones. Overall results indicated that the low level of PIC values observed from the EST-SSR was primary due to the lower number of repeats in an SSR. Therefore, researchers developing EST-SSR should consider to use higher number of repeats and where applicable should choose complex SSRs for their studies.

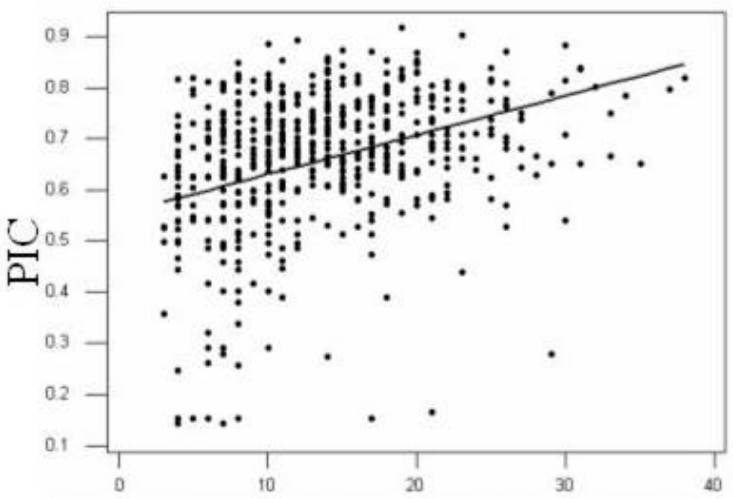

Figure 1 Correlation between PIC and SSR repeat numbers.

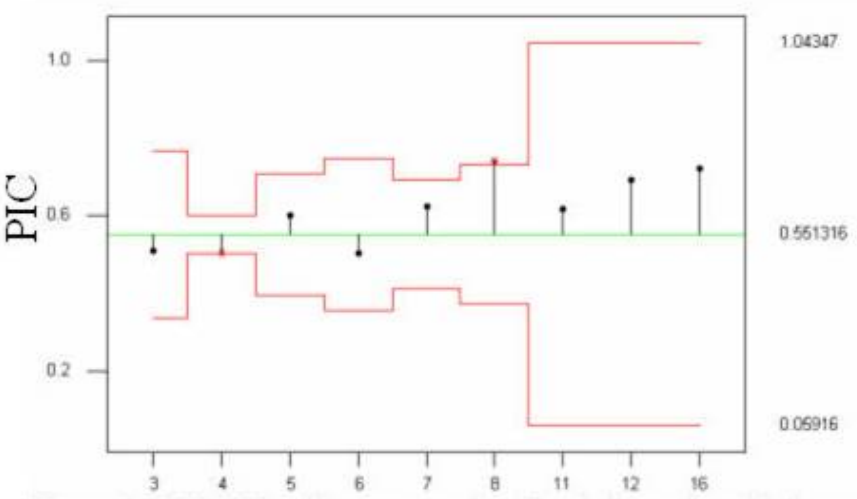

Figure 2. EST-SSR with repeat number 8 and above show higher PIC values.

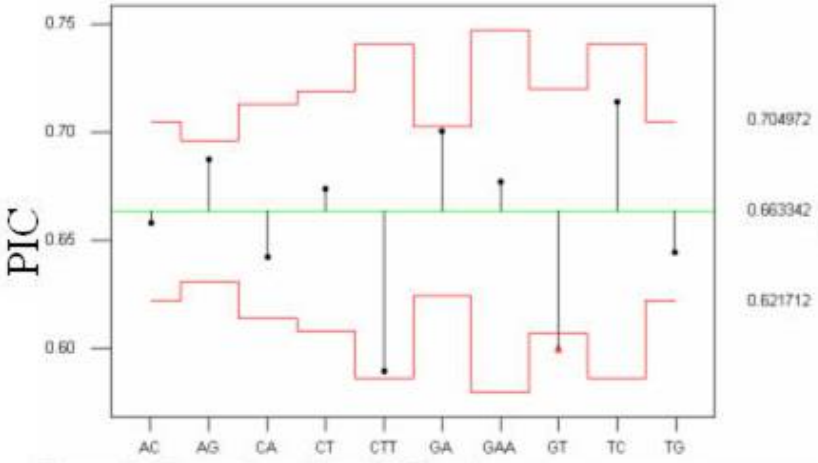

Figure 3. Type of motif and PIC values.

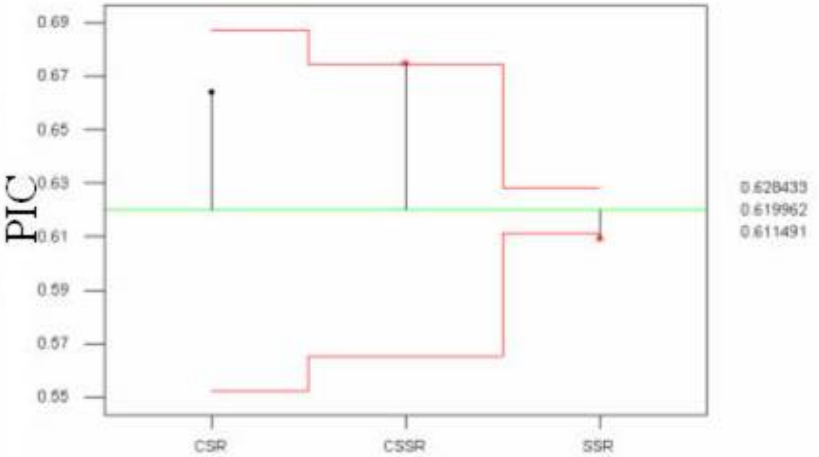

Figure 4. Type of SSRs (simple (SSR), complex (CSSR) and compound (CSR)).

COMPARISON OF EXPRESSION PROFILE OF DIFFERENT PROTEIN CONSTRUCTS

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Overexpression of studied protein in plant is often used for localization studies using fluorescent fusion proteins or GUS as a marker. Alternatively to system in planta, the transformation of tissue cultures or protoplasts could be used as a method of first choice to select constructs for further transformation of plants. The advantage of fluorescent proteins and GUS fused to target protein is their easy detection in microscope. However, constructs often fail to show detectable level of expression. We observed different levels of transient expression in protoplasts using partial and full length constructs of 130 kDa protein fused with YFP. To see if the change of reporter gene or arrangement of construct could lead to a better expression profile, we used various binary vectors to generate a set of constructs with different tags for transient expression in Arabidopsis thaliana and tobacco BY-2 cell cultures. The level of protein expression was monitored by microscopy and molecular-biology methods. As it has been reported, discrepancies in protein expression in vivo could be caused e.g. by the length of a protein, amino acid composition, arrangement of tags (N-terminal, C-terminal). However, main response to construct is given by complex nature of living plant. To find out why the constructs do not work is often time-consuming and majority of laboratories do not report about difficulties experienced. Therefore, studies which deal with these failures and ways to avoid them are rarely available.

Acknowledgement
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MOLECULAR PHYLOGENY OF *Capsicum* COMPLEXES

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Pepper (*Capsicum* spp.) is an important crop grown in the tropical, subtropical and temperate regions. Most of the commercial cultivars of pepper are F_1hybrids of *Capsicum annuum* L. and the use of limited parental lines in hybrid development resulted in increasingly narrower genetic diversity in cultivated peppers. Consequently, the discrimination of commercial pepper varieties from each other or determining the genetic purity becomes more difficult. Although *C. annuum* is considered to be self-pollinating, different rates of out-crossing have been reported. In some cases due to out-crossing identification an individual within a population of a landrace or cultivated varieties of *Capsicum annuum* L. is difficult. The present study examined the genetic identities, within and between species relationship in 24 accessions of 11 species and one inter-species hybrid of *Capsicum* using the random(ly) amplified polymorphic DNA (RAPD)-PCR technique. We used 22 random decamer primers and an average of 5.23 RAPD-PCR amplicons per primer was detected. Using a total of 2760 RAPD-PCR amplicons, principal coordinate analysis (PCO), unweighted pair group mean average (UPGMA) and bootstrap analysis of 2000 replications were implemented using the software MVSP version 3.13O and PAUP version 4.0, respectively.

Accessions *C. annuum* L. [C70-7a, Bye 6787, BP225], *C. galapagoense* Hunz. [AC1501], *C. frutescens* L. [SA36, Heiser 6240], *C. chinense* Jacq. [C334, BP605], *C. baccatum* L. SA219, SA328, C255, *C. praetermissum* Heiser & P.G. Smith C343, *C. tovarii* Eshbaugh, P.G. Smith & Nickrent [BP382], *C. chacoense* Hunz. [SA184, BP618, SA426, BP281], *C. cardenasii* Heiser & P.G. Smith [SA268, E1812], *C. eximium* Hunz [Hawkes 3860], *C. pubescens* Ruiz & Pavon [BP370, C70-12, BP43, BP541] were divided into four distinct groups, corresponding to *C. annuum* complex [C_A], *C. baccatum* complex [C_B], *C. pubescens* complex [C_P] and accessions of *C. chacoense* Hunz. [C_A or C_B] as shown in an UPGMA dendogram (Figure 1). Addition of an inter-species hybrid of *C. baccatum* L. SA219 X *C. eximium* Hunz Hawkes 3860 into the UPGMA and PCO analyses disrupted separation of the 4 complexes combining the *C. baccatum* complex and *C. pubescens* complex. This indicated that the use of 24 accessions of the four *Capsicum* complexes with an unknown accession(s) or variety(-ies) that are under investigation will enhance the correct identification of pepper varieties. In the present study we also investigated the genetic distances between and within the complexes. The overall mean genetic distance (MGD) among the 24 accessions was 0.487±0.082, ranging from 0.877 to 0.316 based on the Jaccard's coefficient. The MGD between the complexes is given in Table 1. The overall results of this present study based on RAPD-PCR suggested that accessions of *C. chacoense* Hunz. [SA184, BP618, SA426, BP281] may be considered as a new complex (*C. chacoense* complex (C_C).

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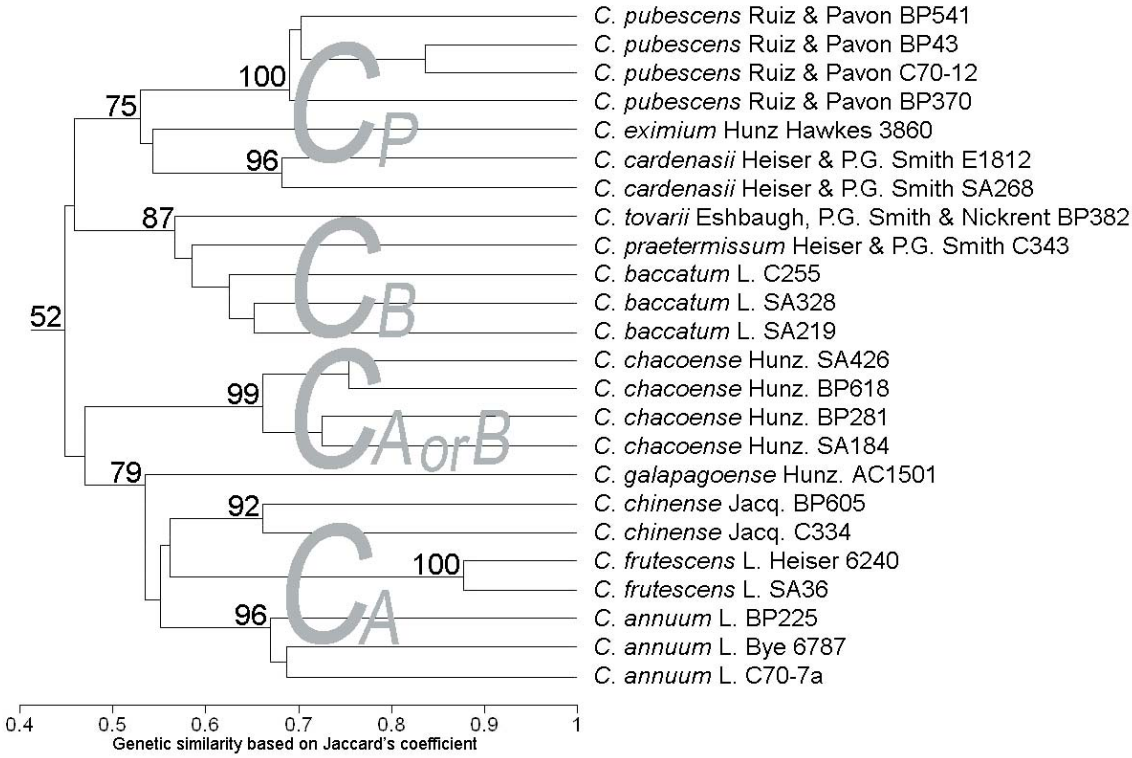

Figure 1. An UPGMA tree of 24 accessions in *Capsicum*. The tree was generated using the Jaccard's similarity matrix. Numbers on branches are bootstrap frequency values for 2000 replicates. C_B, C_P and C_A represent the *C. baccatum* complex, the *C. pubescens* complex and the *C. annuum* complex according to Tong and Bosland (1999), Walsh and Hoot (2001) and Jarret and Dang (2004).

Table 1. The mean genetic distance (Jaccard's coefficient) within and between the *Capsicum* complexes using RAPD-PCR amplicons

		1	*2*	*3*	*4*
1	C_A	0.578 (±0.0798)			
2	$C_{A\ or\ B}$	0.471 (±0.0465)	0.688 (±0.0554)		
3	C_B	0.427 (±0.0333)	0.466 (0.0377)	0.593 (±0.0392)	
4	C_P	0.448 (±0.0474)	0.479 (±0.0514)	0.459 (±0.0409)	0.593 (±0.0954)

DNA METHYLATION STATUS AND FREE POLYAMINES CONTENT IN *Pinus nigra* EMBRYOGENIC TISSUES WITH DIFFERENT MATURATION ABILITY

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Somatic embryogenesis - the formation of embryos from vegetative cells of the plant body *in vitro* - provides an ideal experimental system for the study of fundamental aspects of plant development. For conifers, somatic embryogenesis is also an attractive vegetative propagation method owing to the ability of somatic embryos to regenerate complete plants. In conifer *Pinus nigra* embryogenic tissues have been initiated from immature zygotic embryos and the tissues are being maintained on culture media for long-term cultivation. Plantlet regeneration also occurred in many cell lines, although the maturation of somatic embryos was strongly cell-line dependent (Salajova *et al*., 1999, Salajova and Salaj 2005).

Recently it has been found, the degree of DNA methylation affects organogenesis (Causevic *et al*. 2005) and somatic embryogenesis in some plant species. Santos and Fevereiro (2002) investigated the degree of DNA methylation in the embryogenic and non-embryogenic cell lines of *Medicago truncatula*. The obtained data suggest the production of somatic embryos depends on certain level of DNA methylation. In this context it is interesting to study whether the cell line-dependent maturation capacity in *Pinus nigra* is related to the methylation status of tissues.

In experiments four embryogenic cell lines of *Pinus nigra* initiated from immature zygotic embryos have been included. The cell line differed in micromorphology of somatic embryos as well as in the maturation capacity to produce cotyledonary somatic embryos and regenerate plantlets. The cell line E 181 contained mostly meristematic cell aggregates intermingled by long vacuolised cells and only occasionally bipolar structures resembling somatic embryos. No development toward maturation of somatic embryos occurred in this cell line. Cell lines E182 and 177 contained somatic embryos organized as meristematic cell aggregates connected with long vacuolised suspensor cells without organization into bundles. The maturation capacity was low. Cotyledonary somatic embryos developed in low number but no plantlet regeneration occurred. The cell line E 196 was characterized by the presence of well formed somatic embryos. The embryonal part was composed of tightly packed meristematic cells with regular outline. Long vacuolised cells arranged into bundles were attached to meritsematic embryonal part. Cotyledonary somatic embryos with ability to regenerate complete plantlets differentiated in cell line E 196. In the cell lines with very low embryogenic potential (E 181 and E 177) the percentage of methylated cytosines in genomic DNA was the highest (30 and 32%). With increasing embryogenic potential the percentage of methylated cytosines decreased (22 and 18 %). There is an inverse relationship between free total polyamines content and embryogenic potential of cell lines. In the same way, free spermidine (Spd) content is the highest in embryogenic tissue without regeneration capacity

and the lowest in embryogenic tissue with the highest embryogenic potential. In the cell lines with lower embryogenic ability free putrescine content was low, meanwhile free Spd was the most abundant polyamine.

Acknowledgement
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GRAIN HARDNESS IN WHEAT (*Triticum aestivum* L.): VERIFICATION, DEVELOPMENT AND OPTIMIZATION OF PUROINDOLINE B ALLELE SPECIFIC MARKERS

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Grain hardness (texture) is an important determinant of end-use qualities of wheat (*Triticum aestivum* L.) such as milling yield, break flour yield and starch damage (Hogg *et al*., 2004). Hard wheats produce coarser textured flours with high level of starch damage, better suited for yeast-leavened bread baking, whereas flours of soft wheats, finer-textured with less starch damage, are preferred for cookies, cakes and pastries (Morris and Rose, 1996). Puroindoline genes (*PinA-D1* and *PinB-D1*), located on the *Hardness* locus on chromosome 5DS, form the molecular basis of wheat grain hardness (Law *et al*., 1978; Morris, 2002). The grain texture is soft when both genes are in their functional wild type form (*PinA-D1a and PinB-D1a*). Nevertheless, the failure to express puroindoline a or the alteration of the puroindoline b sequence (predominantly single-nucleotide mutations in *PinB-D1*) result in hard grain texture. To date, twelve alleles from *PinB-D1* have been identified worldwide in hexaploid wheat (reviewed in Chen *et al*., 2006) but only four of them (*PinB-D1a-d*) have been described in European varieties (Huang and Roder, 2005; Lillemo and Morris, 2000).

The aim of our work was the verification, development and optimization of markers that facilitate the differentiation of all puroindoline b alleles identified in European wheat varieties. Ten wheat varieties with defined genotypes (Huang and Roder, 2005) and phenotypes (grain hardness was determined by the particle size index method) were analysed. PCR fragments of *PinB-D1* gene were sequenced from both ends to confirm the SNP (Single Nucleotide Polymorphism) in each genotype selected. To trace the *PinB-D1b* allele, the CAPS (Cleaved Amplified Polymorphic Sequences) technique was performed according to Tranquil li *et al*. (1999). To detect the *PinB-D1c* allele, the CAPS technique was performed according to Lillemo and Morris (2000). The CAPS methods were verified and slightly modified. A new dCAPS (derived CAPS) marker, specific to the *PinB-D1d* allele, was designed and optimized. These markers will be employed in the characterization of selected wheat varieties and genetic resources.

Acknowledgement
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GENETIC MAPPING OF A NOVEL GENE PARTICIPATING IN CHLOROPHYLL BIOSYNTHESIS IN *Arabidopsis thaliana*

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Chlorophyll belongs to a diverse group of molecules with tetrapyrrole structure, the so called porfyrins. Molecules like haem, cobalamin and sirohaem, all having important functions in living cells, also belong to this group of heterocyclic molecules. Chlorophyll, which occurs only in plants and some bacterial species, has a distinct function of capturing the energy of light in the process of photosynthesis. Synthesis of chlorophyll is a complex process consisting of several steps which are catalyzed by multimeric protein complexes or by simple enzymes, in which every protein has its distinct function, generally essential for the right function of the complex. The process of chlorophyll biosynthesis in *Arabidopsis* consists of 14 distinct steps, all of them taking place in chloroplasts, organelles responsible for this process. Disruption of a gene coding an enzyme for chlorophyll biosynthesis leads to an altered phenotype of an afflicted individual. Extensive amount of work has been devoted to identification of genes participating in process of chlorophyll biosynthesis, resulting in knowledge of majority of genes involved. Nevertheless, some unrecognized parts and genes still remain. The screening for mutants defective in chlorophyll biosynthesis have been done in many plant species, the most important ones being *Chlamydomonas* and *Arabidopsis thaliana*.

In our laboratory, a chlorophyll mutant called *chlorominuta* (*chm*) has been obtained during a screening of ethyl methanesulfonate treated seeds of *Arabidopsis* (Nottingham *Arabidopsis* Stock Centre, http://nasc.nott.ac.uk/, catalogue number N246). In this mutant, a chlorophyll defect results in bright yellow phenotype of the plants. Mapping of the mutation was performed on F_2 population. The two parents used in the mapping cross were the mutant plant as a female and a wild-type plant of a polymorphic Landsberg *erecta* ecotype as a male. Only mutant plants of the F_2 population were scored by molecular markers. Single sequence repeat (SSR) and cleaved amplified polymorphic sequence (CAPS) markers have been chosen from The *Arabidopsis* information Resource database (http://www.arabidopsis.org/) including data concerning oligonucleotide sequences. The *chm* mutation was located on chromosome 3 after linkage detection with nga 162 and GAPC markers in position 9.9 ± 3.7 cM. In this chromosomal region none of known chlorophyll biosynthesis related genes has been mapped to date. The closest genetic marker, nga172, was found in the distance approximately 3.20 cM ± 0.28 cM from the gene. The future work is aimed to obtaining a tight linked marker and determining the gene of interest. The molecular base of the mutation will be verified by sequencing.

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MOLECULAR ANALYSIS OF *FRIGIDA* (*fri*) GENE IN LATE-FLOWERING GENOTYPES OF *Arabidopsis thaliana*

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Flowering time is stimulated by both environmental (light, temperature) and endogenous signals. *FLC* and *FRI* genes are the most important endogenous signals controlling onset of flowering in natural populations. *FLC* is a significant flowering repressor under the control of the *FRI* gene. Interaction of both genes is the reason of lateness in natural populations. In the *FRI* gene, two frequent deletions responsible for early-flowering phenotype, L*er*- and Col-type are known. These deletions have been identified only in early-flowering plants but never in late-flowering plants. Therefore, our work was focused on molecular analysis of *FRI* gene in late-flowering ecotypes derived from natural populations in the Czech Republic (*Je-4*, *Je-18*, *Je-27*, *Je-28* and *Hod*) and in four late-flowering mutants *dn* and *L4* (the genetic background Di-G) and *Spi* and *M73* (S96).

For this purpose five PCR primers that cover the complete sequence of the *FRI* gene including the promoter were used. Deletions or insertions were detected using agarose or polyacrylamide gels. Some of the PCR fragments were sequenced.

We confirmed the promoter deletion in early-flowering ecotype L*er*. The same deletion was found also in S96 and Di-G. Surprisingly, we found other larger deletions in the *FRI* promoter of two late-flowering ecotypes (*Je-27* and *Je-28*) and small deletion or insertion in other two ecotypes (*Je-18* and *Hod*). In three late-flowering mutants (*dn*, *L4* and *M73*) we found promoter deletions that were the same as in early-flowering ecotypes. In two late-flowering ecotypes (*Je-27* and *Je-28*) we revealed new changes in the coding region of *FRI* gene such as deletion in the first exon, insertion in the first intron or in the second exon.

Promoter deletions in the *FRI* gene in early-flowering plants are connected with the loss of function (basis of earliness), therefore, we analysed *FRI* expression level by RT-PCR. *FRI* expression in late-flowering plants and, unexpectedly, also in early- flowering ecotypes was found. The results indicate that promoter deletions in *FRI* gene can occur not only in early but also in late-flowering genotypes (ecotypes or mutants) without any impact on *FRI* gene expression and timing of flowering. Our first results also suggest that expression of the *FRI* can be temporarily suppressed during flowering.

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THE USE OF INTER SIMPLE SEQUENCE REPEATS IN BARLEY GERMPLASM COLLECTION EVALUATION

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Inter simple sequence repeats (ISSRs) are the regions situated between microsatellite repeats. The technique is based on PCR amplification of intermicrosatellite sequences. PCR–ISSR is an alternative technique to study polymorphism based on the presence of microsatellites throughout a genom. The aim of the study was to distinguish a set of barley varietes and populations in accord with the genetic dissimilarity based on microsatellite primers. Two ISSR primers were used to analyse 27 spring barley populations (1. Alexis, 2. Lina, 3. Apex, 4. Chariot, 5. Dundy, 6. Landlord, 7. Salome, 8. Brise, 9. Atem, 10. Heris, 11. Ezer, 12. Pribina, 13. Sebastian, 14. Tocada, 15. Carafe, 16. SK 5832-1-00, 17. SK 5835-25-00, 18. SK 4954-7-01, 19. SK 5840-34-01, 20. SK 5976-12-01, 21. SK 57346-00, 22. SK 5398-10-98, 23. SK 5525-1-98, 24. Nadir, 25. Poprad, 26. SK 5374, 27. Diamand). The primers were designed based on trinucleotide tandem repeats and anchored on the 3´ P end HV1 $(CTC)_3GC$ and HV2 $(CTG)_3GC$. A high level of polymorphism was found with both primers, HV1 – 100 % and HV2 – 82 %. In PCR–ISSR analyses, a total of 542 DNA fragments were detected, among which 461 DNA fragments were polymorphic. The PCR–ISSR profiles of two populations (SK 4954-7-01 and SK 5840-34-01) analysed by HV1 primer were identical (DI_{NL}=0,000). By the HV2 primer lower level of genotypes distinction in comparison with HV1 primer was detected. The PCR–ISSR profiles of four pairs populations (Alexis and SK 4954-7-01, Chariot and SK 5840-34-01, Heris and SK 5734-6-00, Brise and Carafe) analysed by HV2 primer were identical (DI_{NL}=0,000).

MOLECULAR ANALYSIS OF CZECH REGISTRATED FLAX VARIETIES

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Flax (*Linum usitatissimum* L.) is self-pollinated plant with 30 chromosomes of about the same size and genome size of 500 Mbp. The objective of this study was to analyze genetic variation of 19 flax varieties currently registered in the Czech Republic. We have used three different molecular fingerprinting techniques (RAPD, SSR, IRAP) based largely on repetetive sequences. RAPD analysis is based on arbitrarily amplified DNA and characterizes nucleic acids without prior knowledge of nucleotide sequence. We have used 12 previously tested RAPD primers, that demonstrated sufficiently high polymorphism in flax. Six of them were adopted from the literature (Fu *et al*., 2005). The number of polymorphic bands ranged from 2 to 9 for target specific primer. None of applied RAPD markers was able to distinguish all tested varieties.

Microsatellites (SSR) are currently highly informative markers due to high polymorphism, reproducibility and codominant character. We have started development of SSR markers according to method of Jakše and Javornik (2001). This method relies on the cutting of genomic DNA with a cocktail of restriction enzymes and the use of long probes for capturing microsatellite-containing DNA fragments. From positive clones, 30 were selected for sequence analysis, from which only 2 sequences contained repetitive motifs. Upon polymorphism testing, one of these markers (*Flax1*) was found polymorphic. Furthermore, additional 20 SSR markers were obtained from gene databases. Ten of them were polymorphic in investigated set and could be used for genotyping of flax varieties. Nevertheless, variability of SSR markers was rather low, with 2-6 alleles per loci.

As retrotransposone sequences dominate eukaryotic genomes and are widely used as universal but specific molecular markers, we have isolated all together 75 different LTR parts of new Ty3-gypsy types elements from flax genome. The designed outwards facing primers were applied in retrotransposone-based IRAP method, which is widely used for fingerprinting. This method relies on knowledge of retroelement sequences, namely long terminal repeats regions (LTR), which are used in specific primer design. On the tested set, 6 IRAP primer combinations produced in average 5 to 10 polymorphic scorable fragments. However even this high polymorphism level did not allowed full identification and distinction, revealing narrow genetic origin of currently grown flax varieties. Further development of both SSR and retrotransposon-based methods is currently performed.

Isolations of flax DNA were carried out using affinity columns Macherey-Nagel. The scored bands were analyzed using software NTSYS-pc version 2.1.

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The list of tested flax varieties

1	Marina	8	Texa	15	Escalina
2	Tabor	9	Super	16	Viola
3	Venica	10	Maryllin	17	Raisa
4	Jordan	11	Agatha	18	Viking
5	Jitka	12	Electra	19	Laura
6	Bonet	13	Ilona		
7	Merkur	14	Diana		

ISOZYME AND DNA MARKERS OF MALE FERTILITY RESTORER GENE IN THE RAPE

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Winter rape (*Brassica napus* L. var. napus) is the most prominent and the most widespread oil bearing plant in the Czech Republic and the second most cultivated crop plant after the grain crops. One of the most effective methods of winter rape breeding is the production of hybrid varieties when male sterile lines and maintaining and restoring lines are progressed. More hybrid systems exist and one of the most worked one is the INRA/Ogura system patented under the marking of OGU-INRA (Vašák *et al*., 1997).

During breeding of winter rape with system OGU-INRA with the use of cytoplasmic male sterility it is necessary to distinguish pollen sterile lines, carrying besides cytoplasmic factors of male sterility recessive allele of fertility restorer gene rfrf, from the fertile lines, carrying at least one dominant allele of the same gene (Rfrf or RfRf). Growing plants to the phase of flowering is very time-consuming, that is why various types of molecular markers were developed. The oldest markers of Rf gene were isozymes of PGI (glucose-6-phosphate isomerase). Isozymes of PGI, belonging to individual lines, differs by electrophoretic spectra – it is possible to distinguish male-fertile from male-sterile plants of rape in early stages of development. Furthermore, homozygous and heterozygous restored plants could be separated too (Delourme and Eber, 1992). In our laboratory, analyses were carried out using vertical electrophoresis on polyacrylamide gel (native-PAGE). Specific detection of PGI isozymes were performed according to formerly published protocol, using immobilized auxiliary enzyme glucose-6-phosphate dehydrogenase (Harrison, 1974). More recently, RAPD (randomly amplified polymorphic DNA) markers, linked to fertility restorer gene, were identified (Delourme *et al*., 1994; Hansen *et al*., 1997). In our laboratory, we tested another 60 RAPD primers, 4 of them enabled distinguishing between pollen sterile and pollen fertile plants. The advantage of RAPD markers is the tight linkage to the restorer gene. On the other hand, the disadvantage of RAPD markers is their dominant character and the lower reproducibility of this technology. That's why the codominant SCAR (sequence characterized amplified regions) markers were developed by sequencing of polymorphic RAPD fragments. We compared six different SCAR markers (four of them were published by Primard-Brisset *et al.* (2005), two of them were developed in our laboratory. The best results were achieved using published French marker SG34.

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ORIGINAL METHOD OF SUNFLOWER BREEDING USING DNA-MARKERS

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Sunflower is an important crop for Ukraine economic. Because of sunflower sown areas extending speeded up breeding process is necessary for creation of competitive hybrids. DNA-marker based selection is one of the power tools for breeding acceleration. DNA-markers could be used to mark a single gene, genome areas or the whole genome. As sunflower is dioecious plant crossing by hand is the bottle neck for the high heterosis level breeding.

The new approach we are developing permits to estimate sunflower lines combinability using DNA-markers with considerable reducing in field research. The first important stage of the approach is preliminarily genotyping of the set of inbred lines that suppose to be investigated for combinability. The next step is a three-stage sowing of mixed seeds. Such three stage sowing process is necessary for effective crosspollination between lines with different vegetation periods. The harvesting should be carried out from the whole crossing plot. Representative sample of harvested seeds should be sown the next year in various environmental conditions. The estimation of plants has to be carried out during all vegetation time and after ripening. The best plants, selected for the desired features, subjected to genotyping and analysis to determine parent lines. The most frequent combination is the best parent lines for desired hybrid.

To examine this approach we have developed multiplex SSR-PCR with 7 microsatellite loci. Ten inbred lines which can be distinguished by genotyping from hybrid pool were selected from a collection. All 10 lines were further crossed according to the full diallele scheme and by the free pollination. Analysis of progeny obtained from these pollinations will be carried out during summer-autumn 2007. It will allow us to estimate whether our new approach really has advantages over the standard breeding scheme.

GENETIC VARIABILITY OF WILD HOPS (*Humulus lupulus* L.) IN CAUCASUS REGION

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Hop (*Humulus lupulus* L.) is a dioecious perennial climbing plant and only female plants are cultivated for commercial use, mainly in brewing industry and to a smaller extent for pharmaceutical purposes. Female inflorescences, referred to as cones, contain hop bitter resins, essential oils, polyphenols and tannins. The origin of the genus is considered to be China (Neve, 1991). Wild hops are distributed throughout the Northern Hemisphere, and have been classified into a number of taxonomic varieties based on their morphology. Historically, hop improvement has been based on European landraces because they provide the flavour qualities preferred by brewers. Wild germplasm provides new genetic resources for breeding to overcome the limited genetic variation present in modern hop breeding programmes. One of the interesting wild hop regions is Caucasus. It is supposed that this region is a half way of cultivated hop from Asia to Europe. In last year, we realized a successful expedition for wild hops in Caucasus region. Wild hops were collected from nine localities: 1 - Vladikavkaz, 2 - river Terek, 3 - Zmenskaja, 4 - Sunža, 5 - Gizel, 6 - Komsomolskoje, 7 - Ursdon, 8 - Craj and 9 - Kabardino-Balkar Republic (Figure 1). The molecular DNA technology is a useful method for the study of genetic diversity, individual genotyping, population structure and phylogeny. We used six SSR (Hadonou *et al*., 2004) and three STS (Patzak *et al*., 2007) loci for molecular analyses of Caucasus wild hops. In our experiment, we used nine Caucasus wild genotypes in comparison to Czech, European and American wild hops. We found there are two different groups of wild hops in Europe and both germplasm were included in Caucasus wild hops. American wild hops were different from all groups.

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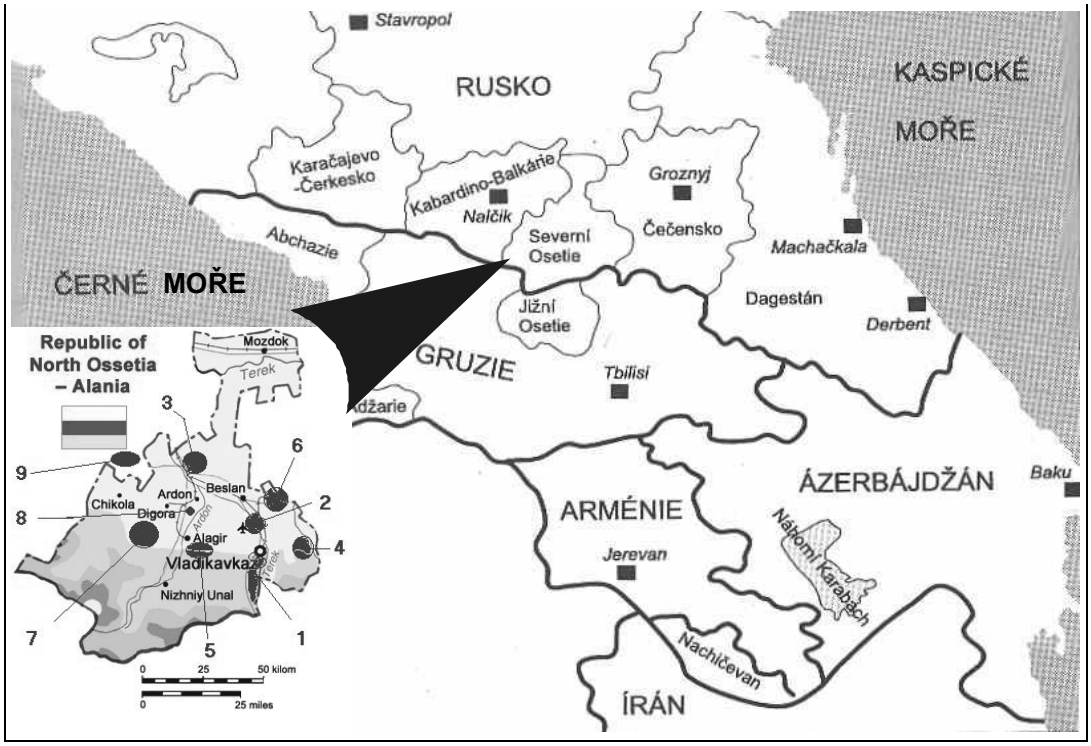

Figure 1. Map of individual localities of wild hops in Caucasus region.

POLYMORPHISM DETECTION IN *IN VITRO* DERIVED *Vaccinium spp.* CLONES

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In *Vaccinium* species, *in vitro* techniques like micropropagation via direct shoot regeneration from existing meristems or *de novo* adventitious regeneration are effective propagation methods for rapid mass production of high quality planting material. However during *in vitro* plant regeneration occurrence of somaclonal variability can be expected. For the confirmation of clonal fidelity of *in vitro* propagated plant material molecular markers are used which enable acceleration and specification in genetic variability testing in comparison with traditional methods based on morphological and physiological descriptors. Selected *in vitro* obtained clones of *Vaccinium corymbosum* L. and *Vaccinium vitis-idaea* L. cultivars, after several months of cultivation on proliferation medium, were used for detection of undesirable genetic variation. Comparison of DNA profiles of *in vitro* derived clones originated from meristems and/or adventitious regeneration was done in selected cultivars.

***Vaccinium corymbosum* L.:**

Cv. 'Berkeley'- one meristem-derived clone (sample no. 1) and three clones derived by adventitious regeneration from leaf tissue cultivated on AN medium with 0.5 mg.l^{-1} zeatin (samples 2, 3, 4).

Cv. 'Bluecrop'- one meristem-derived clone (sample no. 5) and two clones derived by adventitious regeneration from leaf tissue cultivated on AN medium with 0.5 mg.l^{-1} zeatin (samples 6, 7).

Cv. 'Blueray'- one meristem-derived clone cultivated on WPM medium with 0.5 mg.l^{-1} zeatin (sample no. 8).

Cv. 'Darrow '- one meristem-derived clone cultivated on WPM medium with 0.5 mg.l^{-1} zeatin (sample no. 9).

***Vaccinium vitis-idaea* L.:**

Cv. 'Linnea'- one meristem-derived clone cultivated on WPM medium with 0.5 mg.l^{-1} zeatin (sample no. 10).

Regarding morphological characteristics of *in vitro* derived *Vaccinium spp.* plants, no morphological variations and anomalies were observed among different clones. *In vitro* plants were from the beginning characterized by more synchronous growth, formed more shoots, have a bushier growth habit, resulting in a greater number of flower buds. Later no obvious morphological differences were visible among *in vitro*-derived clones and between *in vitro* and by cuttings propagated plants. Regarding fertility and berry quality the plants are also equivalent.

RAPD and flow cytometry analyses were previously used for detection of undesirable genetic variation in micropropagated *Vaccinium* plants (Gajdošová *et al*., 2006). RAPD analysis resulted in characteristic polymorphic banding patterns among cultivars, what enables discrimination of different blueberry cultivars. However, banding patterns were generally monomorphic between maternal and *in vitro* plants originated either via axillary or adventitious organogenesis, what indicates that either no qualitative changes occurred during micropropagation process or more specific DNA markers are necessary to use. By flow-cytometry analysis no changes in ploidy level were found in tested *in vitro* derived clones what confirms that no big changes in

relative DNA content occurred during micropropagation process. Inter-Simple Sequence Repeats (ISSR) markers were also tested for detection of undesirable genetic variation within *in vitro*-derived clones of *V. corymbosum* L. and *V. vitis-idaea* L. (Ostrolucká *et al*., in press). A low DNA polymorphism level and distance index values determined by DNA microsatellite polymorphism have pointed out a stable *in vitro* cultures of *Vaccinium spp.*, however independently from the plant origin. It seems that a primary explant origin might play a role in stability of *in vitro* culture. However, the number of tested samples was not sufficient in order to conclude any definitive statement.

From our and other authors experiences we can conclude that for detection of somaclonal variability in microclonally propagated plants more techniques should be tested, therefore we tried to identify suitable molecular markers for the analysing of clonal conformity for *Vaccinium* species. Retrotransposons as a class of repetetive and temporally mobile sequences are ubiquitous and abundant components of all studied eukaryotic genomes. In higher plants, they constitute more than half of the repetitive DNA, being dynamic genome component by its ability to integrate new copies and facilitate homologous recombination. They show widespread chromosomal dispersion, variable but rather high copy number and random distribution in the genome (cit). Retrotransposons move to new chromosomal locations via an RNA intermediate, and insert new cDNA copies back into the genome. In contrast to animals, plant retrotransposons contain mostly LTR (Long Terminal Repeats) sequences. Retrotransposon families vary in their insertional activity. Such features have directed retrotransposons to become powerful molecular markers for evolutionary, genetic diversity as well as practical studies (Feschotte, Jiang and Wessler, 2002). Particularly long terminal repeat (LTR) class of retrotransposons has driven much attention. For polymorphism detection in *Vaccinium sp*. the universal PBS amplification method of retrotransposon terminal LTR parts was used (Kalendar 2007, in press).

Plant DNA for analyses was isolated from the leaves of *in vitro* plants of 10 different clones at the IPGB SAS Nitra, at the beginning of May 2006. DNAs were precipitated with 1/10 volume of 3M NaAc pH 5.0 + 2.5 volume of 96% Ethanol and sent to the MTT/BI Plant Genomics Laboratory, where a LTRs-type retrotransposons were isolated and later screened for their ability to give the most polymorphic patterns. Several repetitions of PCR with each primer were done, the banding patterns of which were analysed. The DNA analyses were aimed on optimization of PCR reaction by testing of different polymerases (DyNAzyme, FirePol, Biotools, Phusion polymerases) and annealing temperature.

Use of universal retrotransposon amplification method of retrotransposon terminal LTR parts for polymorphism detection in *Vaccinium sp*. tissues show many polymorphisms between somatic lines and their source. Several primers were proved to be very suitable markers for *Vaccinium* genetic characterization and they can be used not only for *in vitro* clone characterization, but also for evaluation of genetic variability of natural populations of *Vaccinium sp.* what is very important for Finland and Slovakia, where *Vaccinium sp.* are wide spread.

Acknowledgment

DNA analyses were realized at the Institute of Biotechnology, MTT/BI Plant Genomics Laboratory, and University of Helsinki, Finland. The work was supported by Slovak Grant Agency VEGA, project no. 2/5128/25 and COST 863

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PLENARY SESSION III:
GENETIC TRANSFORMATION STRATEGIES FOR PLANT IMPROVEMENT

FUNCTIONAL GENOMICS OF DROUGHT RESPONSE IN CEREALS

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Breeding for yield stability under water limited conditions plays an essential role in the reduction of economic and social consequences of global climate changes. We show that two exotic drought resistant genotypes (Kobomughi and Plainsmann) differ in root growth rate, root/shoot ratio, and adaptation to low soil water content. These genotypes exhibit characteristic transcript profiles as shown by barley macroarray studies using 10500 unigenes. Reprogramming of gene expression primarily occurred during the 1-2 weeks of water stress, and 6.1% of tested genes were up-regulated in roots of the more adaptive Plainsmann plants. The time course for expression of gene clusters from Kobomughi genotype revealed a prompt and transient gene activation that can help the survival of plants through function of various defense mechanisms. The aldo-keto reductases (AKRs) can detoxify lipid peroxidation products (4-hydroxynon-2-enal) and glycolysis-derived reactive aldehydes (metylglyoxal) that contribute significantly to cellular damages caused by variety of environmental stresses such as drought, high light intensity, UV-B irradiation, cold). Overproduction of AKRs in transgenic tobacco or wheat plants provides considerable stress tolerance and resistance to methylglyoxal. Molecular and physiological characterization of transgenic material will allow to use AKR-based detoxification system in the improvement of stress adaptation of crop plants.

TOPOGRAPHY FACTORS INFLUENCING THE QUANTIFICATION OF GFP FLUORESCENCE IN LEAVES OF TRANSGENIC TOBACCO AS REVEALED BY IMAGE ANALYSIS

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During the process of integration of foreign genes into plant genome, the assessment of the performance of a foreign gene in recipient plant is highly desirable. In the experiments aimed at the qualitative improvement of various plant traits *via* insertion of one or a few genes, the content of a new, recombinant, protein is one of the main concerns of plant transgenosis. Beside the exact methods of direct determination of the protein content, indirect estimations based on the performance of a linked marker gene(s) is available and in many cases reliable and sufficient enough.

Here we present the attempts to relatively quantify the performance of the green fluorescent protein (GFP) marker in the leaves of transgenic tobacco, based on the measurements of its fluorescence. Tobacco (*Nicotiana tabaccum*) is still one of the main model species used in the plant transgenesis experiments. Transgenic plants were obtained after *Agrobacterium tumefaciens* (LBA 4404 strain) mediated transfer according to the standard co-cultivation protocol (Horsch *et al*., 1985) and regenerated as mentioned in Hraška *et al.* (2005). We utilised the stereomicroscope Leica MZ 12 fitted with GFP excitation/ emission filters coupled with CCD camera. The fluorescence was measured with Lucia® software and obtained mean brightness (MB) values (relative fluorescence units) were used for relative quantification of the transgene performance (Hraška *et al*., 2005). With the regard to the reliability, all measurements for each sample were triplicate and the average MB values were subjected for statistical assessment using the Statistica® software.

In our first experiment (1), we have just verified if such approach is useful or not. Statistical analyses of MBs obtained from leaves of *in vitro* cultivated control and PCR-verified (data not shown) transgenic plants confirmed, that such attempt is possible, nevertheless revealed some variability of the GFP fluorescence in studied samples within one leaf (Fig. 1a). The most pronounced were the differences between the leaf mesophyll and conductive elements (midrib). Moreover, since we have studied *in vitro* leaves at the stage of 3-4 true leaves only, questions regarding the influence of the sample physiological age and topography within the mature leave have arisen. Therefore, more precise studies of the GFP fluorescence patterns regarding all these issues were performed, with the aim to define the fluorescence patterns within mature leaves/ plants and to obtain more reliable results. For these experiments, transgenic plants of T_1 generation were used. First, based on our previous observations, the influence of leaf surface (leaf orientation) on the quantification of GFP fluorescence was studied (2). Obtained results confirmed, that the fluorescence detected on the abaxial side is more in-

tensive than on the adaxial side, although the data obtained from the abaxial side exhibited higher variability (Fig. 1b). The purpose of our next experiment (3) was to study the variability of detectable fluorescence within each particular leaf and to define GFP fluorescence pattern within the mature leaf surface. Using a corc borer, leaf discs from eight defined positions were taken, covering the whole leaf surface (Fig. 2a) and subjected for fluorescence measurements using the same approach as mentioned above. Obtained data confirmed the presence of variability in detected fluorescence within the each leaf, when the strongest signal was found in a leaf tip area (Fig. 2b). Next, in the consistency with our previous experiment (1), the influence of strongly fluorescing conductive elements, namely footstalk (midrib), on a yield of the fluorescence from neighbouring mesophyll tissue was recorded. This resulted in a relatively high variability of the data obtained from such positions even among the leaves detached from the same plant. This trend was also evident in all studied samples. Nevertheless, the main trend in the fluorescence pattern shown on the Fig. 2b was same for all studied samples.

Using the equipment available in our laboratory, comprising of the GFP observation/ detection device and the software for image analysis, we applied a simple and rapid method for relative quantification of the GFP fluorescence in leaves of transgenic tobacco. Using this methodology, it is possible to assess the regenerated primary transformants and select the most favourable individuals already in early stages of their regeneration. There is no evident obstacle for using the same approach for the assessment of the marker gene performance in the mature plants/ leaves for various purposes, but the above mentioned factors should be taken into account when selecting the tissue samples and finally interpreting the obtained data.

Acknowledgements

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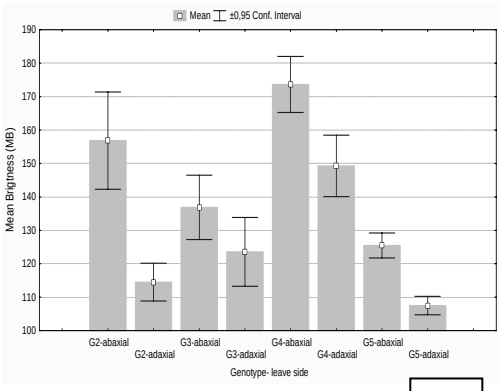
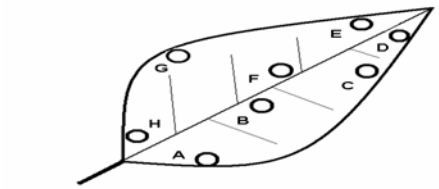
Plant number	1*[12a]	2*[12b]	3*[17]	4*[17b]	5*[18]	6*[18a]	7*[18b]	8*[22a]	9*[22b]	10*[7]	11*[8b]	C
MB from the medial part of leaves (including the midrib)												
MB	134.03 ±26.00	11.92 ±5.11	226.55 ±24.29	13.58 ±4.74	87.72 ±26.19	7.18 ±5.05	143.85 ±57.09	95.86 ±43.52	78.14 ±9.36	1.12 ±0.17	134.98 ±23,79	0.91 ±0.14
MB from the periphery of leaves (excluding the midrib)												
MB	137.16 ±15.82	11.12 ±2.02	219.38 ±14.60	13.92 ±3.09	46.16 ±6.76	18.64 ±7.75	86.04 ±14.45	55.46 ±10.36	47.73 ±7.69	1.33 ±0.20	69.49 ±10.74	0.47 ±0.11

1a

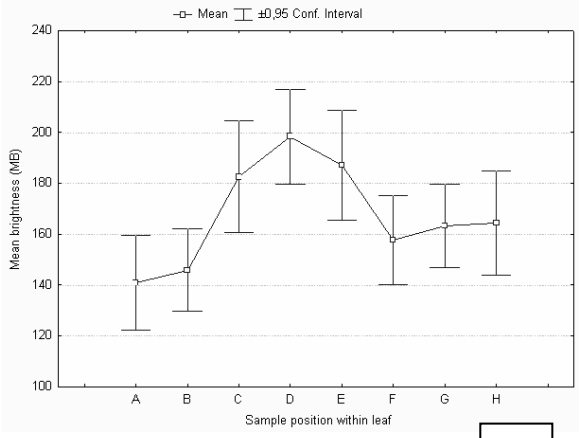


1b

Figures 1a, b. Comparison of the GFP fluorescence variability within the abaxial leaf surfaces involving/ excluding the midrib part of different transgenic lines Nos. 1-11. Each data set represents an average MB value from particular location obtained from 3-4 leaves (**a**). Comparison of the fluorescence intensity between the abaxial and adaxial sides of a leaf (**b**). Weaker GFP fluorescence was detected on the adaxial side of leaves detached from all investigated T_1 plants. Each bar represents the average MB value obtained from three leaves, eight measurements were performed on each leaf. Average MB values for particular position obtained from all investigated tissue samples are given here. From each plant, at least four leaves were investigated and MB values from defined positions were detected. In both experiments, the fluorescence of control samples expressed as MB value was always bellow 30. Index data with asterisks (**a**) represent the original labelling of primary regenerants.



2a



2b

Figures. 2a, b. Schematic drawing of tobacco leaf showing the locations of samples used for the fluorescence measurements. Eight distinct positions cover the whole leaf area (**a**). Comparison of the fluorescence intensity among the eight defined positions (A-H) within the leaf surface (**b**). The increase of the GFP fluorescence intensity on abaxial side of leaf tips was evident in all investigated T_1 plants. Other legend as in Fig. 1b.

EXPRESSION PATTERNS OF ENDO-β-1,4-GLUCANASES IN *Arabidopsis* ANALYZED BY HISTOCHEMICAL GUS-STAINING

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Plant endo-glucanases have been suggested to be involved in a number of physiological processes. In Arabidopsis, this gene family is composed of 25 members, which are divided into three subfamilies (Figure 1). The α and β subfamilies contain proteins with a putative signal peptide and they are predicted to be secreted. The γ subfamily is composed of three proteins (KOR, KOR2, KOR3) with membrane spanning domain (Libertini *et al.*, 2004). So far, a putative function has been suggested only for the three KOR proteins and one of the secreted glucanases.

Twenty-one promoter region of *Arabidopsis* endo-β-1,4-glucanase genes have been amplified, cloned into the binary vectors, mobilized in *Agrobacterium tumefaciens* and transformed into the *Arabidopsis* plants with the aim to (1) gain insight into the function of individual gene in cell processes, (2) to determine the degree of overlapping expression of duplicated genes and (3) to identify specifically the gene(s) involved in pod dehiscence. As expression vector was used the binary vector pCAMBIA1301, where the promoter regions were cloned at the 5' end of the intron-containing β-glucuronidase (gusA) gene.

Histochemical GUS-staining analysis of different parts of plants showed, that some of the analysed endo-glucanase genes are more specific than other. The results of the GUS-staining pattern were correlated with the expression patterns of each of the Arabidopsis endo-glucanase genes in the GENEVESTIGATOR database.

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EFFICIENT TRANSFORMATION OF *Lotus corniculatus* CV. BOKOR USING THE SUPER-BINARY *Agrobacterium tumefaciens* VECTOR AND GA_3 - STIMULATED MULTIPLICATION OF TRANSFORMED SHOOTS

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Bird's foot trefoil (*Lotus corniculatus* L.) is a perennial forage legume, which in many areas replacing alfalfa, for its tolerance to adverse environmental conditions and high nutritive value. It has been used as a model plant for nodulation and nitrogen fixation competence and condensed tannin metabolism studies. The productivity and value of bird's foot trefoil could be increased by the introduction of stable inherited traits such as pest, disease and herbicide resistance or improved protein quality. All of these traits are not available in natural *L. corniculatus* populations, but current advances in biotechnology approaches provide a potentially powerful tool for achieving these goals. The prerequests for the transfer of foreign genes into any plant species by genetic engineering are an efficient gene delivery system, effective selectable marker for transformed cells, and the ability to easy regenerate transgenic plants from transformed tissue. The local cultivar Bokor (Mijatović *et al*., 1986), selected by the polycross method in the Center for Agricultural and Technological Research in Zaječar (eastern Serbia), was used in this study. This cultivar displays a high regeneration potential *in vitro* and satisfactory field performance of regenerants (Nikolić *et al*., 1997). It is susceptible to genetic transformation with *A. rhizogenes* (Nikolić *et al*., 2003/4). We present here the efficient transformation of the Bokor cultivar with *A. tumefaciens* super-binary vector. The hygromycin-resistant transgenic plants were obtained by direct organogenesis from cotyledonary explants for five months. Cotyledonary explants derived from 31 6-day-old seedlings were inoculated with *A. tumefaciens* strain LBA4404 carrying super-binary vector pTOK233 (Hiei et al., 1994) with *hpt*, *nptII*, *uidA*-intron genes and extra set of *vir* B,C and G genes. For shoot regeneration explants were cultivated on MS medium with BA and NAA (0.5 mg l^{-1}, each) for 20 days, followed by transfer to MS hormone-free medium. Sixty five percent of explants produced shoots 25 days after inoculation by *Agrobacterium*. Five percent of inoculated regenerating explants survived five subculture on selective media with increased concentration of hygromycin (5-15 mg.l^{-1}), while all the control explants were died. Finally 52 hygromycin-resistant shoots, originated from four genotypes, were obtained (Table 1) and efficiency of transformation was 42 %. Regenerated shoots were rooted on MS medium with IBA 0.2 mg.l^{-1}. The presence of GUS-intron gene in T_0 plants was confirmed by the histochemical GUS assay and PCR analysis. With the aim to improve growth and multiplication, the nodal segments of the transformed shoots were incubated on GA_3-medium (0-4 mg.l^{-1}) for 20 days, and then returned to MS hormone-free medium for two subculture periods. GA_3 induced abundant *de novo* shoot regeneration on callus tissue (Fig. 1) and increased the number of axillary shoots (Fig. 2). It has been concluded that (1) *A. tumefaciens* LBA4404/pTOK233 super-binary vector would be the recommendable vector for *L. corniculatus* transformation; (2) Multiplication of transformed shoots could be improved by nodal segment culture on GA_3-containing media.

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Table 1. Transformation efficiency and shoot regeneration in cotyledonary explants of *L. corniculatus* cv. Bokor.

Treatment	No. of isolated explants	No. (%) survived regenerating explants*	No. of survived shoots**	Transformation efficiency (%)***	No. (%) of rooted plants
LBA4404/pTOK233	124	6 (4.9)	52	42	38 (73)
Control	28	0	0	0	0

** Survived cotyledonary explants after five subculture on hygromycin-containing media*
*** Survived shoots after seven subculture on hygromycin-containing media*
**** Transformation efficiency = (No. of survived shoots/No. of isolated explants) x 100*

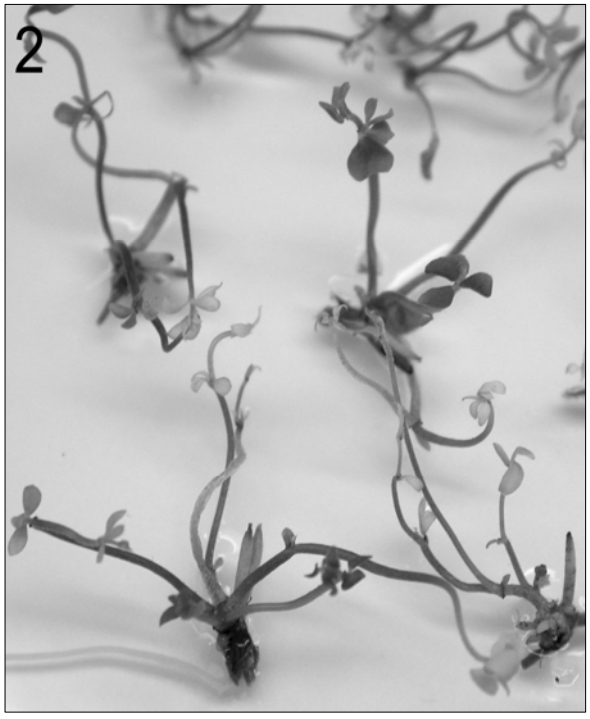

Figure 1. *De novo* shoot regeneration on callus tissue developed on nodal segment of transformed plant cultured on medium with 1 $mg.l^{-1}$ GA_3

Figure 2. Axillary shoot multiplication in nodal segment of transformed plant cultured on medium with 2 $mg.l^{-1}$ GA_3

TRACKING OF THE CAMV 35S EXPRESSION PATTERN IN THE FLOWERS AND REPRODUCTIVE ORGANS OF GFP TRANSGENIC TOBACCO REVEALED ITS HIGH ACTIVITY IN VASCULAR TISSUES

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Insertion of various genes into plant genome is a very promising way to modify various desirable plant traits. The use of a suitable promoter is a tool for desirable direction of expression of newly inserted gene. Although a wide range of various promoters is available nowadays, constitutive promoters such as *Cauliflower mosaic virus* promoter CaMV 35S are still widely used in the transformation experiments. Despite the fact, that the CaMV 35S is generally considered as a constitutive promoter, some reports suggests, that it is not always truth and many reports concerning this issue are available (Williamson *et al*., 1989; Yang and Christou, 1990; Malik *et al*., 2002). Studies of the promoter expression in plants were usually performed using some marker genes, especially *uidA* and localisation of GUS product were preferred. Nevertheless, these GUS assays suffer from some limitations and therefore the use of some other marker for such purposes is highly desirable. Based on its unique properties, the green fluorescent protein (GFP) (Prasher *et al*., 1992) seems to be more suitable tool for tracking the promoter activity in the plant tissue rather than the GUS (Stewart, 2001; Hraška *et al*., 2006). Here we present original data obtained based on a study of the CaMV 35S expression in flowers of transgenic tobacco plants using the GFP, since this organ was not of main concern of other reports. Moreover, we have used transgenic plants of two subsequent generations, with the aim to track the stability of the promoter performance in two distinct generations.

Transgenic plants were obtained as described in Hraška *et al*. (2005) and T_1 and T_2 plants were tested for the presence and expression of the *gfp* gene using the PCR, dot-blot hybridization and RT-PCR. Detached inflorescences and mature flowers in various stages of development were immediately investigated under the fluorescence microscope Leica MZ 12 fitted with GFP excitation/emission filters (Hraška *et al*., 2005). Usually, the studied samples were moistured by distilled water to prevent the desiccation. Photographs were taken with the Olympus digital camera C5050 Zoom.

The presence and expression of *gfp* gene in randomly selected individuals was confirmed by the above mentioned molecular methods (data not shown) and these plants were used for following microscopic studies. Immature and mature inflorescences were detached from glasshouse cultivated T_1 and T_2 plants and subsequently investigated. First, whole unopened and fully opened inflorecences/flowers were investigated. GFP fluorescence was observed along the whole organ surface, with a very intensive fluorescence associated with vascular tissue within the petals (Fig. 1). Sepals exhibited weaker fluorescence as compared to petals, but such fluorescence was also consistent during the whole flower surface and existence. Nevertheless, the distinct fluorescence was observed in the trichomes, frequently occurring on the sepal surface (Fig. 2). Next, inflo-

rescences were investigated using the longitudinal cuts and reproductive organs were of main concern. The most intensive GFP fluorescence at the beginning of flower/ inflorescence development was visible in the ovary, and such fluorescence was retained during the further development, till the flower maturity (Figs. 3, 4). In addition, strong fluorescence, and therefore CaMV 35S activity was evident in the stamen filaments, continuing as conductive elements inside the anthers. Such strong fluorescence was easily visible also in the anther body (Fig. 5, 6). This strong fluorescence was retained from early developmental stages, which we were able to study, till the flower maturity. Other reproductive organs such as anthers, stamens, and styles exhibited weaker GFP fluorescence compared to ovary and filaments, but still intensive enough to permit the CaMV 35S tracking. On the other hand, compared to all previously mentioned organs, stigma exhibited very weak fluorescence, indicating also the low promoter activity (Fig. 7). Developing sepals were always easily visible and observed pattern was constant during the flower development, indicating also constant promoter activity in this floral part (Figs. 8, 9, 10).

The GFP marker has been utilised as an efficient tool for the tracking of CaMV 35S expression in the flowers of transgenic tobacco and unique data regarding this plant part are presented here. As expected, distinct fluorescence patterns were revealed between various parts within the inflorescence and also mature flowers. Surprisingly, high levels of the GFP fluorescence and thus CaMV35 S activity were detected in various vascular tissues (within the petals, stamens and anthers), and therefore higher promoter activity in this tissue type within reproductive organs and flowers could be hypothesised. Moreover, since the same patterns were observed in the samples detached from both, T_1 and T_2 generations of plants, the stability of the CaMV 35S expression among various generations can be concluded. To our knowledge, this is the first detailed report about the behaviour of CaMV 35S promoter in flowers and reproductive organs, performed on two subsequent generations of autogamized transgenic plants.

Acknowledgements

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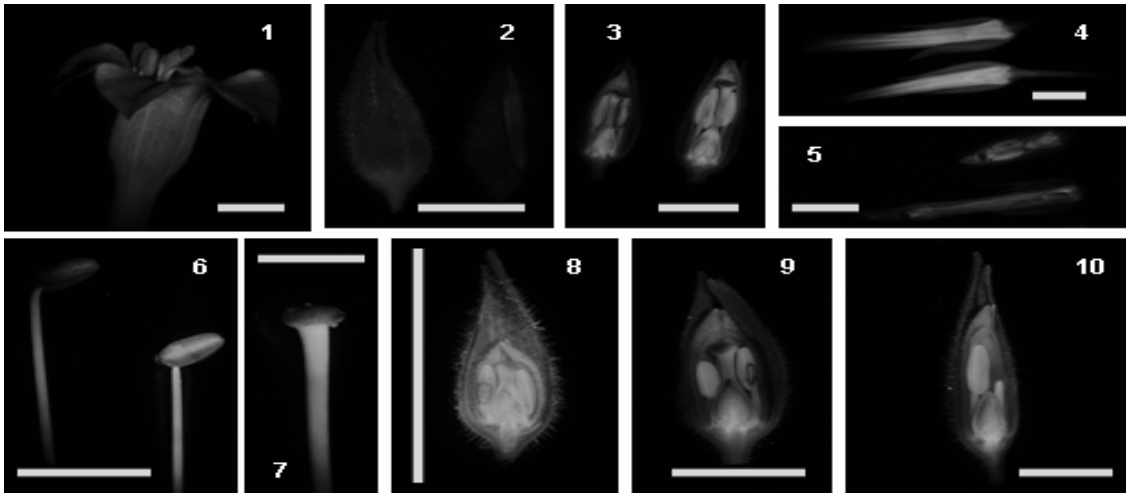

Figures. 1-10. Expression patterns of CaMV 35S promoter driven GFP synthesis in flowers and generative organs. **Figure 1** is a fluorescence image of the whole mature flower. Note, that an intensive fluorescence in the vascular tissue is easily visible. **Figure 2** is a fluorescence image of the immature inflorescence. Note, that sepals possess weaker fluorescence compared to petals (**Figure 1**). **Figure 3** is a fluorescence image of transversal cut alongside the immature inflorescence of a different developmental stage. Intensive fluorescence is visible in ovary, anthers and stamen filaments. **Figure 4** shows the fluorescence pattern in the basal part of inflorescence before the opening while **Figure 5** shows the fluorescence patterns in the reproductive organs of the inflorescence in the same stage as shown on the **Figure 4** (bottom, the upper sample is only for demonstration that a stable fluorescence pattern is retained throughout the organ development). **Figure 6** demonstrates an intensive fluorescence and thus assumed performance of the CaMV 35 within the vascular tissue of anthers, while on the other hand, a low activity of the promoter can be assumed based on the GFP fluorescence in the stigma (**Figure 7**). **Figures 8** to **10** illustrate the GFP fluorescence pattern within the developing inflorescence. An intensive fluorescence is apparent in the ovary, anthers and petals. Sepals are distinguishable well, but the intensity of a green fluorescence is lower as compared to other floral parts, mainly petals. All figures are fluorescence images of the samples detached from GFP transgenic plants. Light-blue bars represent 1 cm.

ROOT REGENERATION IN TRANSGENIC GRAPE ROOTSTOCK 'RICHTER 110' CARRYING THE *ROL*B-GENE FROM *Agrobacterium rhizogenes*

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Breeding of grape rootstocks pursues two main goals: (i) protection against grape phylloxera, a root destroying insect, and (ii) improving adaptability to a wide range of soil and climatic conditions. Apart from the long testing required, breeding of new rootstock cultivars through crossing is complicated by the high degree of heterozygosity in grapes. Alternatively, gene transfer permits addition of single traits, largely without affecting the genetic background. Here we report on *in vitro* rooting performance in transgenic grape rootstock 'Richter 110' (*Vitis berlandieri* × *V. rupestris*) carrying the *rol*B gene from *Agrobacterium rhizogenes*. *Rol*B has been choosen by us as a model, since it has been useful in promoting rooting in other woody fruit crops (*e.g.*Welander *et al*., 1998; Zhu *et al*., 2003) and, as suggested in *rol*B-transformed *Rhododendron*, also might improve adaptability to adverse soil conditions (Dunemann *et al.*, 2002).

Transformation was achieved by co-cultivation of anther-derived somatic embryogenic callus with *Agrobacterium tumefaciens* LBA4404 harbouring plasmid pHrB. The T-DNA of pHrB contains the *HPT* gene, conferring hygromycin resistance, as selectable marker, and the *rol*B gene under control of its own promoter. After at least six passages in the presence of hygromycin, thirty resistant clones were selected from co-cultivated calli, seven of which have been examined in more detail up to now. By PCR using transgene-specific primers it was revealed that *HPT* is present in all seven clones, while only six clones possess the *rol*B gene. Rooting performance *in vitro* was examined using tip, node and internode segments of *in vitro* grown shoots as explant materials. Cultivation was performed on MS medium (Murashige and Skoog, 1962; macronutrients at half strength) with different auxin and/or cytokinin supplements. Number of primary roots, total length, surface and/or volume of roots per explant were determined and evaluated statistically. In general, root number and other traits correlated positively. Compared to internode segments, clone-specific differences were less significant in tip and node segments, due to positional effects and higher variation within variables. In different experiments, *e.g.* the one illustrated in Fig. 1, internode segments of four transgenic clones produced consistently more and longer roots than those of non-transgenic control. Clone HrB-5.1 behaved variable, while the other two remaining transgenic clones were similar to non-transgenic control. Among these two was clone HrB-2.2 which doesn't possess *rol*B. The most notable feature of 'high rooting' transgenic clones is their ability to root early and profusely in the absence of auxin. Moreover, presence of 0.25 µM IAA generally didn't improve rooting in those clones, while being strongly promotive in non-transgenic control. Experiments to study rooting and growth under various stress regimes *in vitro* and *in vivo* are currently being set up.

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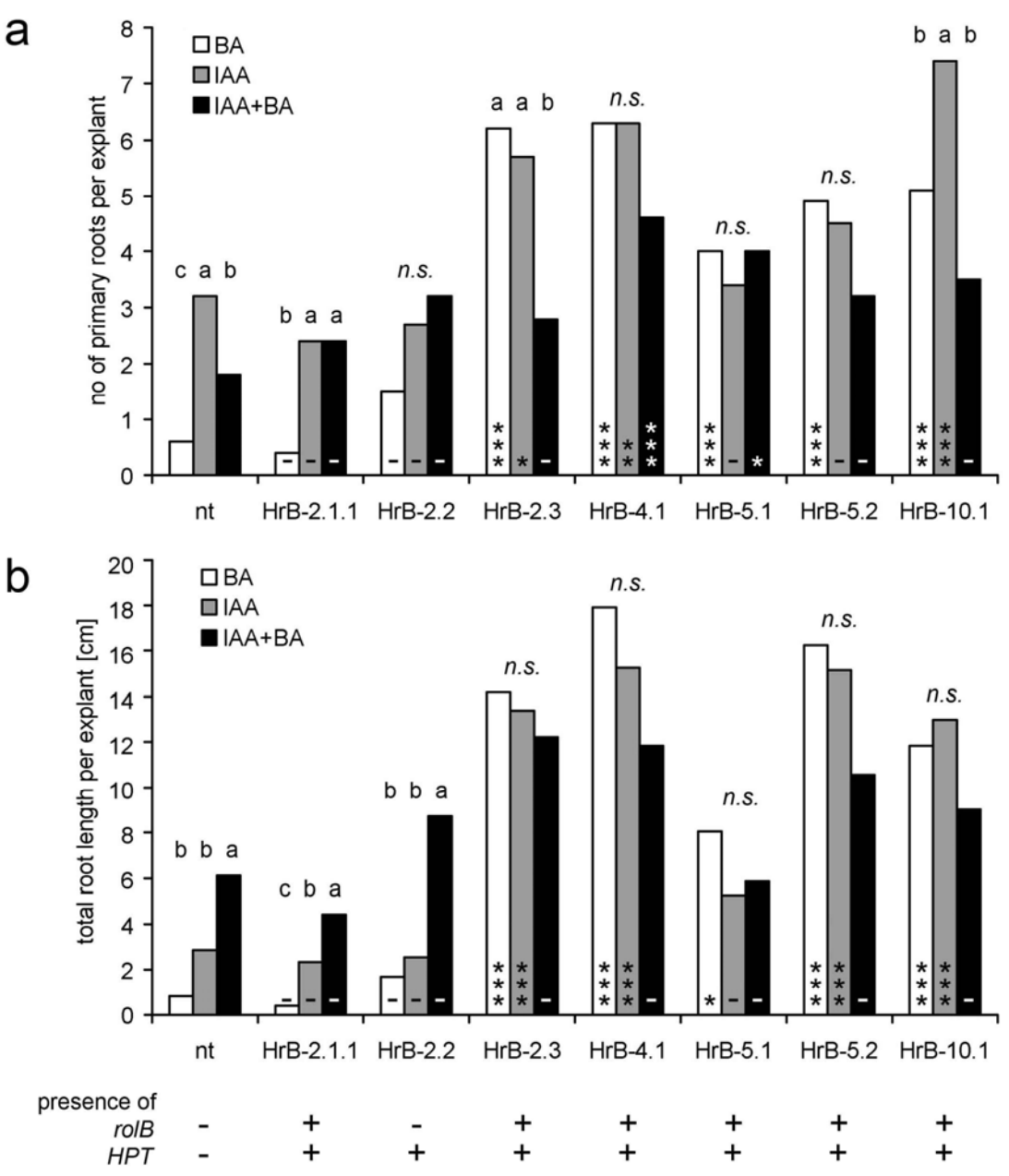

Figure 1. *In vitro* rooting of internode segments taken from non-transgenic (nt) 'Richter110' and seven transgenic clones (HrB-2.1.1 till -10.1). Explants were cultured in the dark at 24 °C in the presence of 0.2 µM BA, 0.25 µM IAA, or 0.25 µM IAA + 0.2 µM BA, respectively. After 17 weeks, numbers of primary roots per explant were counted (**a**) and total root lengths per explant were determined using WinRHIZO software (**b**). To reveal significant differences between culture media, mean values were compared separately within each clone using Tukey's test (different letters above columns indicate significant differences at p<= 0.05; *n.s.* = not significant). In order to reveal significant differences between nt and HrB clones, mean values of respective media were compared using Dunnett's test (marks inside columns: - not significant; * significant at p<=0.05; ** significant at p<=0.01; *** significant at p<=0.001).

STABLE TRANSFORMATION OF EMBRYOGENIC TISSUES OF HYBRID FIRS BY *Agrobacterium tumefaciens*: GUS EXPRESSION IN REGENERATED TISSUES AND DEVELOPING SOMATIC EMBRYOS

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In recent years reliable protocols have been developed for gene transfer of several conifer species using biolistic system or *Agrobacterium*-mediated transformation (Minocha and Minocha, 1999).) The efficient transformation requires reliable regeneration system allowing plant regeneration from transformed tissues (Holland *et al*., 1997). For conifers somatic embryogenesis is considered as an effective regeneration system (Cyr and Klimaszewska, 2002) and as a rule embryogenic tissues are often involved in genetic transformation experiments. Somatic embryogenesis is the process of formation of somatic embryos *in vitro* and in conifers the process is mostly initiated from juvenile explants. Somatic embryos are capable of maturation and finally plantlet development.

In hybrid firs *Abies alba* x *A. cephalonica* and *Abies alba* x *A. numidica* embryogenic tissues were initiated from immature zygotic embryos (Salajova *et al*., 1996). The embryogenic tissues produced somatic embryos that developed on maturation medium and regenerated plantlets (Salaj *et al*., 2004). The aim of the presented work was genetic transformation of mentioned embryogenic tissues using *Agrobacterium tumefaciens*.

For experiments cell lines AC 78, AC 2 (*Abies alba* x *A. cephalonica*) and AN 72 (*Abies alba* x *A. numidica*) were selected. In the time of experiments the tissues were cultured on medium DCR (Gupta and Durzan, 1985) containing 6-benzyladenine 1 $mg.l^{-1}$. Before transformation procedures the tissues were cultivated on media with different concentrations (0, 5, 10, 15, 20, 25, 30, 35 $mg.l^{-1}$) of geneticine, to test their ability to grow later on selection media. Geneticin at concentration 10 $mg.l^{-1}$ suppressed the growth of tissues and was chosen for selection after co-cultivation with *Agrobacterium tumefaciens*. The co-cultivation occurred during 48 hours with *Agrobacterium tumefaciens* (strain AGLO) containing the plasmid pTS2 carrying *gus* and *npt* II genes. Following the co-cultivation, the tissues were transferred to medium containing 300 $mg.l^{-1}$ claforan and a week later to selection media.

Approximately one week after cultivation on selection media, the cultures started to grow, and the regenerating tissues were observed as small clumps, reaching the size 1-3 mm. The regenerated resistant tissues were transferred to fresh selection media in three-week's intervals.

Following two months of selection, the resistant tissues were tested for GUS activity by histochemical approach. For both cell lines, most of geneticin-resistant sublines showed GUS-positive response (11 out of 14 for AN 72 and 10 out of 16 for AC2). Intensive blue coloration was observed mainly in the meristematic embryonal part of somatic embryos. The control tissues (without co-cultivation) showed negative response, i.e. no GUS-like staining. Genomic DNA from transformed and un-transformed (control) tissues was subjected to PCR analysis to detect the presence of T-DNA in putative transformed tissues. The samples from transformed tissues gave predicted DNA fragments bands of 320 bp for *gus* gene and 280 bp for *npt* II gene, whereas no amplification was detected in samples from un-transformed tissues.

Following the molecular analysis, the transformed tissues were cultured on maturation medium, with the aim to obtain cotyledonary somatic embryos and regenerated plantlets. DCR medium (Gupta and Durzan, 1985) containing abscisic acid (10 $mg.l^{-1}$), polyethylene glycol-4000 (10%), sucrose (3%) and gelified with gelrite was used for maturation of somatic embryos. Within 6-7 weeks cotyledonary somatic embryos developed and were screened for GUS activity. After histochemical staining, blue coloration was observed thorough the whole body of somatic embryos. The somatic embryos germinated and regenerated small plantlets. Molecular analysis of cotyledonary somatic embryos and regenerated plantlets is being in progress.

Acknowledgement
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THE INFLUENCE OF SELECTED COCULTIVATION TREATMENTS ON *Agrobacterium*-MEDIATED GENETIC TRANSFORMATION EFFICACY OF PEAS

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Agrobacterium-mediated genetic transformation includes several methodological steps. Among them, the co-cultivation step is a crucial period when the two different biological elements i.e. plant explants, and *Agrobacterium* share the same space and conditions. Many parameters should be tested to satisfy both partners and to guarantee a successful outcome (Hansen and Wright, 1999). During co-cultivation, the conditions (time period, temperature, light/dark period, pH, presence of various chemical additives, concentration of *Agrobacterium* in solution) may vary and significantly affect the final efficacy of transformation process.

Material and Methods. Cultivars of dry-seed peas (*Pisum sativum* L.) - Adept (yellow seed, leaf type), Komet (yellow seed, leaf type), Menhir (yellow seed, semi-leafless type) were used in a set of experiments with application of various cocultivation substances, such as acetosyringone (AS), L-cysteine (CYS), dithiothreitol (DTT), glutathione (GLU), macerating enzymes – cellulase (CEL) and pectinase (PEC). The effect of continuous light and dark conditions during co-cultivation were tested as well. Basic *Agrobacterium*-mediated transformation was done as in Švábová *et al.* (2005) with a hypervirulent strain of *Agrobacterium tumefaciens* EHA 105 with plasmid pGT89 carrying reporter *gus-int* gene and *bar* selectable marker gene for phosphinothricin resistance with double 35S promotor. Putative transformants were assayed histochemically for GUS, the explants were classified in four coloration categories (0-1-2-3). PCR proof was done in a set of randomly taken samples to verify transgene integration, and corresponded with positive GUS staining. The data were analysed via frequency distribution of explants in coloration category (%) and further processed by analysis of variance.

Results and Discussion. The concentrations of AS 100 and 200 μM were applied in agreement with experiments dealing with other leguminous plants (French beans – Dillen *et al.* (2000), Zambre *et al*. (2003), soybeans - Santarém *et al.* (1998), Ko and Korban (2004), azuki bean - Yamada *et al*. (2001). The concentration of acetosyringone which was proved as the most effective was 100 μM, in case of 200 μM we recorded slight decrease of the number of positively GUS stained explants (Fig. 1).

In preliminary experiments, CYS was applied to co-cultivation media in concentrations 300, 600, and 900 mg/l, similar to that ones for soybean by Olhoft *et al.* (2001a), but without any positive effect. Further, the concentrations were lowered to 100, 200, 300 $mg.l^{-1}$, and the lowest concentration pointed to slight increase in number of positively GUS stained explants. Then, another - still lower concentration sequence was prepared with 0, 25, 50, 75, 100 $mg.l^{-1}$. The average frequency of non-transformed explants obtained from all cultivars (Adept, Komet and Menhir) after treatment with CYS oscilated between 70 and 77%, as compared to control 85% (Fig. 2).

DTT was employed in concentrations 0, 0.25, 0.50, 0.75, 1 μM. All cultivars responded to DTT concentrations very mildly, moreover in all experiments the control variant possessed the highest frequency of positively GUS stained explants. In the highest DTT concentration (1 μM) the percentage of negatively stained explants raised up to 84%.

Experiments with GLU treatment were set in two basic variants (0; 0.20; 0.40; 0.60 μM, and 0; 1; 10; 100; 1000 μM). First set examined lower concentrations, similarly to some experiments on peanut by Quisheng *et al.* (2005), in the second set, the concentrations were increased according to the paper of Olhoft *et al.* (2001) who examined

1mM concentration on soybean. In both experimental sets, the frequence of GUS positive explants has not significantly changed after the treatment with GLU in all examined concentrations.

Macerating enzymes were applied in two series of experiments with CEL and PEC in concentrations 0; 0.05; 0.10; 0.20. Finally, the combinations of (A) 0.10 % CEL and 0.05 % PEC and (B) 0.20 % CEL and 0.20 % PEC similarly to the recommended concentrations in sunflower (Weber *et al*., 2003) were examined. The results figure that all combinations have negative effect on the transformation efficacy. In control variants, 20 days after treatment about 40-50 % explants were positive in GUS tests. The variants treated with the enzyme mixtures (A and B) resulted in deep fall in percetage of GUS positive explants.

Further series of experiments were carried out with two light/dark regimes. The variant co-cultivated in darkness (24 h) slightly increased the number of negative (GUS 0) plants as compared to the co-cultivation in continuous light. Moreover, in continuous darkness the explants did not exhibit uniform *uidA* expression, while in continuous light 15 % of explants were scored by evaluation category 3, e.g. the most intensive display of GUS staining (Fig.3).

Summarised, among all studied co-cultivation substances, the positive effect was recorded only for acetosyringone (in 100μM concentration) and L-cysteine (0.25-0.75 $mg.l^{-1}$ of co-cultivation media). Glutathione was without any positive or negative result, athough the experiments were repeated, and two different combinations of concentration variants were used. Dithiothreitol and both macerating enzymes (which were used separately, as well as in two combinations) had negative effect on frequency of GUS positive explants. Continuous light during co-cultivation possessed positive effect on the frequency of explants uniformly expressing GUS. Statistical analyses did not prove any significant differences.

Acknowledgment

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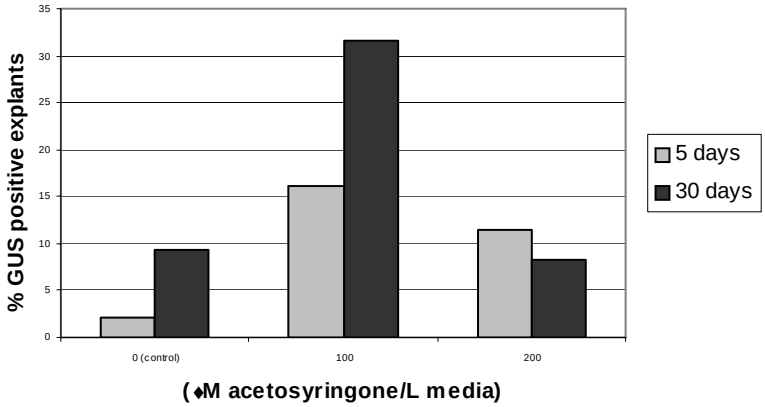

Figure 1. The effect of acetosyringone on GUS expression for cv. Adept 5 and 30 days after co-cultivation

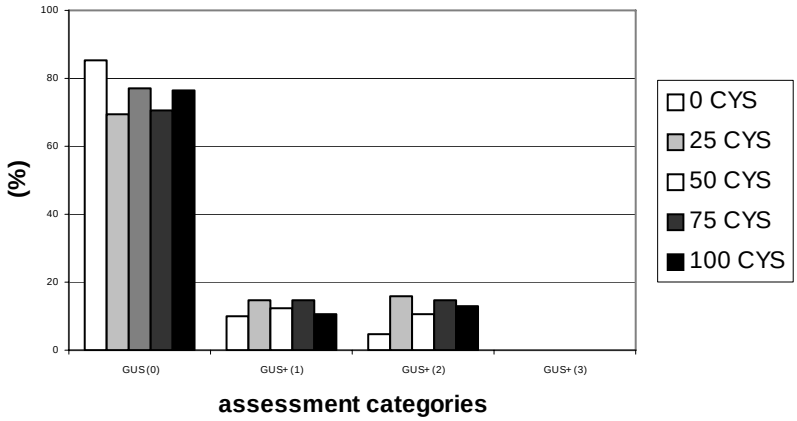

Figure 2. Frequency distribution of GUS positive explants after L-cysteine treatment (pooled data for cvs. Adept, Menhir and Komet)

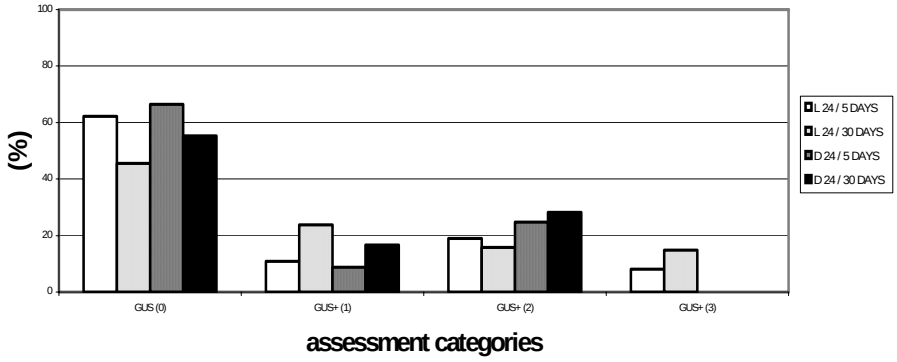

Figure 3. The influence of continuous light (L24) and dark (D24) conditions during co-cultivation on the frequency distribution of explants in evaluation categories in GUS testing (%) after 5 and 30 days from transformation event

EXPRESSION ENHANCEMENT OF EPITOPES OF HUMAN PAPILLOMA-VIRUS IN PLANTS

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The influence of synergistic interaction between PVX-based vector and *Potato virus Y* (PVY^O) on the level of transiently expressed protein consisting of PVA CP with two different epitopes from HPV (L2ACPE7) was examined in four different hosts - non-transgenic *N. tabacum* and *N. benthamiana* and trangenic *N. tabacum* expressing potyviral P3 and *N. benthamiana* expressing HC-Pro proteins.

In our experiments, co-infection with PVX-PVY^O viral combinations in *N. tabacum* plants caused a high increase (50%) in amount of expressed L2ACPE7, in comparison to control plants infected with L2ACPE7 only. Our experiments involving mixed infection of PVX and PVY^O in tobacco plants prompted the hypothesis about suppression of PTGS, a plant antiviral defence mechanism that normally limits the accumulation of infecting viruses (Marathe *et al*., 2000). We do not observe any changes in expression levels of L2ACPE7 in tobacco plant transformed by P3 protein of PVA compared with nontransgenic tobacco.

In *N. benthamiana* HC-Pro plants double infected with PVY^O and PVX, we detected by anti-PVA CP antibodies the highest increasing (100%) of expressed L2ACPE7 in comparison to control plants infected with L2ACPE7 only. We also detected the increase of expressed protein by anti-L2 antibodies (20%). Our data indicate that high expression levels of the PVA HC-Pro in transgenic *N. benthamiana* together with the HC-Pro provided directly from the PVY^O infection multiplied in their effects. In our opinion this cooperation is possible because of very low sequence homology of these HC-Pro proteins, or we can take in account that the HC-Pro expressed in transgenic plants is not identical to the HC-Pro produced during potyviral infection (Savenkov and Valkonen, 2001). This interaction of different HC-Pros can enhance the level of heterologous protein expressed from vector based on unrelated plant viruses, e.g. the potyviral HC-Pros mediate the synergistic effect on *Potexvirus* (PVX).

Acknowledgement

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THE BENEFITS OF TREE BIOTECHNOLOGY TO SCIENCE AND SOCIETY

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Tree biotechnology offers the potential to introduce genetic gain into forest trees, thereby improving wood quality characteristics such as timber stiffness and pulpability, as well as providing better growth and form and improved manufacturing and appearance characteristics. Further, biotechnology can introduce traits that act to counter environmental challenges such as insect and pathogen attack, drought and adverse climatic conditions. New traits can be introduced either for tree improvement purposes, or for studying their function and interaction with the environment.

The scientific benefits from tree biotechnology stem from a detailed knowledge of tree genomics and gene expression profiles. Further, scientific evidence around long term stability of novel gene expression in trees will enhance our ability to use transgenic trees in commercial plantation forestry. Environmental impacts research ensures the safety and sustainable use of modified trees.

Societal benefits arise largely from the substantial contributions that forest trees make to both the environment, and to economic growth. Economic benefits are becoming increasingly obvious in particular when tree biotechnology leads to the deployment of highly improved trees in plantation forests. These trees show much enhanced productivity compared to natural stands and they will increasingly provide and sustain the resources required to satisfy the ever-increasing demand for wood world wide. The productivity of plantation forests is much greater than that of natural stands, making them an essential and renewable resource for timber and wood products, while also reducing the demand for further exploitation of natural forests. By doing so, they become key in protecting biodiversity worldwide. Further, recent discussions around climate change and CO_2 sequestration indicate that trees, and in particular those enhanced using biotechnology, will make a substantial contribution to stabilising the climate through their ability to sequester carbon. In future also, trees are expected to play a substantial role in replacing fossil fuels for the production of bioenergy and as biomaterial feedstocks for biorefineries.

THE APPLICATION OF THE SEED SPECIFIC CRUCIFERIN PROMOTER IN SELF-EXCISION CRE/*LOX*P STRATEGY

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At present a strong effort is focused to develop strategies for efficient elimination of selectable marker genes after selection transgenic plants before their introduction into environment. Designed inducible auto excision Cre/l*ox*P strategy involves induction of cre recombinase by specific cruciferin C promoter from *Arabidopsis thaliana* during the seed development and following excision of cre recombinase and selectable marker *ntp* II gene, both placed between directed oriented lox sites in T-DNA. Despite the fact that cruciferin C promoter is considered to be tightly seed specific, the phenomena of premature auto excision was observed in all transformants probably either due to some exogenous stimuli or putative read through effect. However, the running excision event was also confirmed in all developing seed of tested single T-DNA copy transgenic tobacco line T_0-30 using semi quantitative PCR. Although efficiency of auto excision event was lower as was expected, 10.2 % of F1 progenies were revealed as marker-free.

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ENVIRONMENTAL SAFETY OF GENETICALLY MODIFIED CONIFERS

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Tree biotechnology provides an opportunity to increase genetic gain in plantation forestry, in particular, where traits not readily available in the breeding population can be transferred into already superior genotypes. However, genetic engineering has been criticised for introducing a new level of risk and uncertainty with regard to the environmental impacts of genetically modified tree plantations. Using a field test with genetically modified radiata pine, impacts on soil microbes and invertebrates in New Zealand have been studied. To date, microbial populations have not shown any significant sign of impact from genetically modified trees and horizontal gene transfer could not be detected. Further, invertebrate populations associated with transgenic trees in the field appear unaffected.

Further studies have focussed on the continued expression of novel genes in trees that were transformed using a Biolistic® transformation technology. Gene expression has so far continued in up to 8 year old trees in a field test and in up to 6 year old trees in the glasshouse. Gene silencing effects were detected in very young trees of up to 6 months, but never in older trees growing in the field or in glasshouses.

The results of these studies will be discussed in the context of the potential deployment of genetically engineered trees in plantation forestry.

TIME TO RELAX GMO REGULATION IN EUROPE

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The European regulation of biotechnology crops is most stringent in the world. It seriously hampers progress in agriculture. Sadly not only in Europe, but also in several developing countries, which need to improve their food production. In addition European legislation is wasting money for unnecessary procedures. There is enough experience gained during ten years of GM crops application to seriously evaluate the ratio of risk to benefit. This evaluation is the key step in the approach of EU. Legislation asks of the assessment of risk when GM crops are used. It does not evaluate the risk of the alternative situation when GM crops are not used. The precautionary principle is applied only to GM crops application, never to alternative solutions of, e.g., pest control. Very often the precautionary principle is misused as a gate for fabrication of catastrophic scenarios. These are used to manipulate the public. It was demonstrated by the Eurobarometer 2005 how such propaganda inseminates public opinion with shameful nonsense.

There are many voices asking for the change of this politics. Most important come from the European Parliament, British ACRE, EuropaBio and other European sources, but also from Africa and other developing countries. Facing the strong support of biotechnology in China and other Asian countries as well as in Latin America, Europe has highest time to seriously thing over its approach to biotechnology in agriculture.

POSTERS

DETECTION OF METALLOTHIONEIN LEVEL AT TRANSGENIC PLANTS OF TOBACCO

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Heavy metals are one of the most toxic and undesirable compounds polluting environment. As trace elements, some heavy metals (e.g. copper, selenium, zinc) are essential to maintain a metabolism. However, other ones such as cadmium, lead, and mercury are toxic at all. At higher concentrations both groups of heavy metals (toxic and essential) lead to poisoning. Plants protect themselves against heavy metals via synthesis thiols rich peptides such as glutathione or phytochelatins. These peptides are not as effective as animal heavy metal protective proteins, which is called metallothioneins (MT). These proteins belong to group of intracellular, low molecular and cysteine-rich proteins with molecular weight from 6 to 10 kDa. MTs consist of two binding domains (α, β) that are assembled from cysteine clusters. The N-terminal part of the protein is marked as α-domain, which has three binding places for divalent ions. β-Domain (C-terminal part) has the ability to bind four divalent ions of heavy metals. In the case of univalent ions of heavy metals, MT is able to bind twelve metal ions. The aim of the present was to characterize constructs of plants capable of synthesis of MTs.

The model plant tobacco (*Nicotiana tabacum*) was chosen to be transformed by *Agrobacterium tumefaciens* (Macek *et al*., 2002). The construct were then able to express yeast metallothionein gene originating from *Saccharomyces cerevisiae*. Nevertheless we were interested in the issue, if we would be able to quantify the expressed MTs. For these purposes we suggested and optimized electrochemical method called adsorptive transfer stripping technique coupled with differential pulse voltammetry - Brdicka reaction (Petrlova *et al*., 2006). We have optimized following experimental conditions: the temperature of supporting electrolyte - 5 °C, the time of accumulation of MT - 120 s, the concentration of $Co(NH_3)_6Cl_3$ - 1 mM. Under these most optimized conditions we analysed samples of transgenic plants. We found out that plant were able to synthesized from tens to hundreds ng MT per g of fresh weight.

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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF TRANSGENIC *Hypericum perforatum* L. PLANTS

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The high demand for pharmaceutical compounds from *H. perforatum* has promoted an effort to search, along with traditional ways, for biotechnological approaches towards an increase of their content. Production of secondary metabolites can also be affected by *Agrobacterium*-mediated transformation. Recently the efficient hairy root induction and plant regeneration in *H. perforatum* using the natural vector system of *Agrobacterium rhizogenes* has been reported (Di Guardo *et al*., 2003). The aim of this work was to characterize hairy root-regenerated plants of *H. perforatum* derived from *A. rhizogenes*-mediated transformation of root cuttings at morphological and biochemical levels in *in vitro* and in *ex vitro* settings.

The appearance of hairy root-regenerated *in vitro* grown plantlets of *H. perforatum* was as that typical for plants transformed by *A. rhizogenes* T-DNA (or its part). The number of hypericin-containing glands per mm^2 of leaf lamina was much higher in transformed clone. The typical feature of transgenic clone was abundant root formation with reduced geotropism. The content of hypericins was significantly increased in transformed plants.

After successful adaptation to *ex vitro* conditions, transgenic plants exhibited bushy dwarfed phenotype in the first and second year of *ex vitro* cultivation. Number of dark glands per leaf was reduced in transgenic plants, but dark gland density was higher comparing with control plants. Transgenic plants had fewer flowers which were much smaller. Flowers of transgenic and control clones contained equal number of floral whorls, except for stamen number reduced in transgenic clone. Floral parts of transgenic clone were significantly smaller than those of the control clone. Density of dark glands was higher on petals of transgenic plants. While control plants produced seeds, transgenic ones were sterile. In contrast to *in vitro* culture, the content of hypericins in plants cultivated *ex vitro* in transgenic clone was significantly lower.

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TRANSFORMATION OF A NICKEL HYPERACCUMULATING ECO-TYPE OF *Allysum murale* WALDST. ET KIT.

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Shoot cultures of nickel hyperaccumulating eco-types of *Allysum murale* were established *in vitro* from epicotyl explants of seedlings aseptically germinated on hormone-free MS medium. For shoot multiplication the effect of 0-0.5 $mg.l^{-1}$ kinetin was investigated. Kinetin at 0.1 $mg.l^{-1}$ although providing only 2.3 shoots per explant was considered as optimal since higher kinetin concentrations induced vitrification.

Bacterial inoculation was performed with overnight suspension of *A. rhizogenes* A4M70GUS which containing GUS gene co-integrated in plasmid pRiA4. Shoots at first or second internode above the medium were wounded by a sterile needle dipped in bacterial suspension. After 30 days 25% of 124 inoculated epycotile explants produced roots on the wounding site. Roots were excised and each was propagated further on hormone-free medium as separate clone (31 different clones). In the first passage clones 3 and 6 could be distinguished from the others due to their fast growth and spontaneous shoot regeneration (Fig. 1). On the same media roots consisting clones 2, 12, 23, 24 and 25 produced calii. Cytokinins supplemented as 0-2.0 $mg.l^{-1}$ BA or 1.0-3.0 $mg.l^{-1}$ TDZ promoted also shoot regeneration in clones 7, 12, 23, 24 and 25. Most clones regenerated shoots on media with 1.0 $mg.l^{-1}$ BA. Cytokinins stimulated development of callus or even root elongation in some clones. Histological analysis of shoot regeneration revealed it to be either direct or indirect. In direct regeneration shoots developed directly from root tissues with little adjacent callus formation. In indirect regeneration shoots differentiated from callus developing on roots. All regenerated shoots were very short and vitrified and they were therefore further culture on hormone-free medium until normal growth resumed.

Regenerated shoots from clones 3, 6, 12, 23 and 25 after two passages on hormone-free medium resembled plants of normal phenotype. They were further cultured on media with 0.1 $mg.l^{-1}$ kinetin same as non-transformed shoots used in studies on heavy metal hyperaccumulation. Shoots of transformed clones were characterized by good elongation and lateral shoot branching, short internodes, minute slightly curled leaves and well developed plagiotropic root system spreading over the surface of media. Thus plants which spontaneously regenerated from *A. rhizogenes* transformed *A. murale* manifest a characteristic Ri syndrome phenotype. Shoots of all five clones had a positive GUS reaction. Transformation was confirmed by PCR analysis using (Fig. 2).

In a separate treatment lots consisting 10 transformed and 10 non-transformed clone 6 shoots, 12-15 mm in length were cultured together in 100 ml wide neck Erlenmayer flasks containing 40 ml medium supplemented with 0.1 $mg.l^{-1}$ kinetin and 1, 4 or 8 mM $NiCl_2 \times 6H_2O$. According to this investigation transgene shoots hyperaccumulate nickel same as the non-transformed controls (Table 1.). Increase of nickel content in the medium equally decreases shoot multiplication and elongation and increases leaf chlorosis both in transformed and non-transformed plants.

In non-transformed shoots inhibitory effect of nickel is apparent already at the lowest nickel concentration. Transformed shoots produce abundant root system and the

inhibition of their development begins at higher nickel concentrations. This observation indicated *A. rhizogenes* transformed plants of *A. murale* could be used for phytoremediation instead of standard non-transformed plants.

Table1. Effect of $NiCl_2$ on multiplication and elongation of *A. rhizogenes* transformed and non-transformed shoots after 35 days

$NiCl_2$ x $6H_2O$ mM	Length of shoots (mm)		No. of lateral shoots	
	non-transform.	transformed	non-transform.	transformed
0	27.7 ± 1.5	21.3 ± 1.1	2.3 ± 0.2	4.2 ± 0.4
1	23.2 ± 1.4	17.0 ± 0.9	2.5 ± 0.4	3.7 ± 0.4
4	19.6 ± 0.6	17.4 ± 0.8	1.9 ± 0.2	2.8 ± 0.3
8	16.7 ± 0.6	14.5 ± 0.4	1.3 ± 0.2	2.1 ± 0.3

Figure 1. Spontaneous regeneration of shoots from hairy root cultures of clone 6

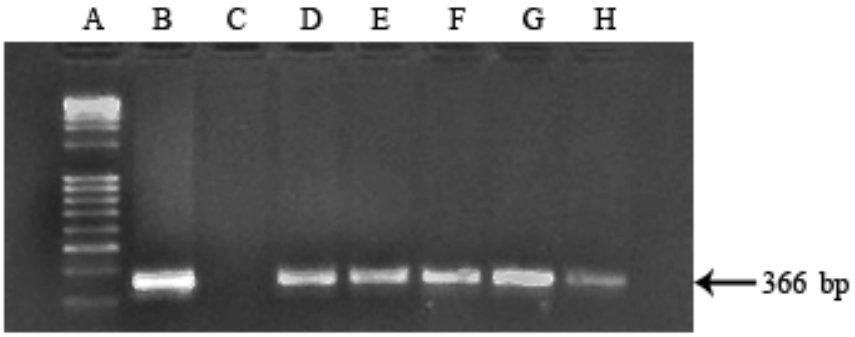

Figure 2. PCR analysis of *A. rhizogenes* transformed shoot cultures: lines 1- 1 kb DNA ladder, lines 2- positive control (A4M70GUS), lines 3- negative control (non-transformed shoots), lines 4-7- transformed shoots clones 3, 6, 12, 23, 25 respectively.

DETECTION OF CHITINASE ACTIVITY IN VARIOUS TISSUES OF CARNIVOROUS PLANT *Drosera rotundifolia* L.

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Plant chitinases belong to relatively large gene family subdivided in classes that suggest class-specific functions. They are commonly expressed upon viral, bacterial and fungal infection, by various stress signals such as wounding, drought, cold ozone, heavy metals as a part of plant defence mechanism. Moreover, chitinases have important non-defensive functions in growth and development process. Although an endogenous substrate for plant chitinases has not yet been found, it was suggested that they catalyze the hydrolysis of β-1,4 linkages in chitin, a homopolymer of *N*-acetyl-D-glucosamine. In general chitinases are categorized into three principal types: endochitinases which hydrolyze chitin randomly at internal sites, exochitinases (also called chitobiosidases) which catalyze the release of diacetylchitobiose unit from chitin chain and *N*-acetalglucosaminidases (chitobiases) which act in exo-splitting mode on diacetylchitobiose or higher analogs of chitin

To gain better understanding of the role of chitinases in carnivorous plant *Drosera rotundifolia* L., different plant tissues were subjected to molecular-biochemical analyses. *In situ* hybridisation with a 325 pb *DrChit1* fragment (GenBank AY622818) as a probe, revealed the presence of chitinases in all analysed tissues (leaves, stems, flowers and roots) of *Drosera rotundifolia* L. The chitinolytic activities were determined using three substrates: glycol chitin, synthetic 4-methylumbelliferyl-β-D-*N,N´*-diacetylchitobioside [4-MU-$(GlcNAc)_2$] and 4-methylumbelliferyl-β-D-*N,N´,N´´*-triacetylchitotrioside [4-MU-$(GlcNAc)_3$]. The chitinases in all crude protein extracts isolated from flowers, stems, leaves and roots, were active against both fluorescent substrates [4-MU-$(GlcNAc)_2$] and [4-MU-$(GlcNAc)_3$]. In case of endochitinase activity for short oligomers [substrate 4-MU-$(GlcNAc)_3$], the highest activity was detected in roots while in flowers reached approximately 40%, in stems 21% and in leaves 16% of activity measured in roots. To identify these chitinolytic enzymes further, the crude proteins from analysed organs were separated on a 12.5% SDS-PAGE. Following renaturation, the proteins were probed with substrates: glycol chitin, fluorescent 4-MU-$(GlcNAc)_2$ and 4-MU-$(GlcNAc)_3$. These results showed the presence at least four chinolytic enzymes with chitobiosidase (chit 1), endochitinase for short oligomers (chit 2) and endochitinase for long chitin polymers (chit 3, chit 4) activities in all analysed organs of sundew.

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OPTIMISING THE CONDITIONS FOR SUCCESSFUL *Agrobacterium*-MEDIATED TRANSFORMATION OF WHEAT

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Wheat has been considered as most recalcitrant species in *in vitro* regeneration and genetic transformation. Important advances have been achieved using the microprojectile bombardment and first fertile transgenic wheat plants were obtained by Vasil *et al.* (1992). Recent reports have demonstrated that *Agrobacterium*-mediated transformation offers a better alternative than biolostic gun for transgenes delivery to wheat. It is a method of choice for low copy insertions of defined fragments of T-DNA with minimal rearrangements and makes the possibility of transferring larger DNA segments into recipient cells. In our previous study (Mitić *et al.*, 2004) we have successfully transformed immature embryos of spring wheat cultivar Vesna at an average efficiency of 0.41 % using the super-binary vector in *A. tumefaciens* strain LBA4404. The obtained data suggest that the transformation of cv. Vesna is feasible but the low transformation frequency represents the major concern and need to be improved. In this part of experiments we varied the factors that could be produced significant differences in T-DNA delivery and regeneration. The freshly isolated immature embryos of spring wheat cultivar Vesna as well as the immature embryos precultured four days on MS medium containing 2 $mg.l^{-1}$ 2,4-D were used as explants (Table 1). The explants were inoculated with *A. tumefaciens* strain LBA4404 carrying super-binary vector pTOK233 (Hiei *et al*., 1994) with *hpt*, *nptII*, *uidA*-intron genes and extra set of *vir* B,C and G genes and cocultivated in the presence of 100 $mg.l^{-1}$ ascorbic acid as antioxidant. The presence of induction agent acetosyringone (100 μM) in the inoculation and cocultivation medium was varied (Table 1). The selection procedure was followed: one subculture (20 days) on medium with 10 $mg.l^{-1}$ hygromycin, one subculture on medium with increased (20 $mg.l^{-1}$) hygromycin concentration and then three subculture on 10 $mg.l^{-1}$ hygromycin containing media. GUS expression was histochemicaly detected in immature embryos and immature embryos-derived calli 3, 6 and 25 days after inoculation and in regenerated plantlets survived selection procedure. Inclusion of ascorbic acid in cocultivation medium increased the immature embryo response after cocultivation in comparison to previous experiment when ascorbic acid was omitted. The presence of acetosyringone in the inoculation and cocultivation media improved β-glucuronidase expression observed in precultivated immature embryos. When acetosyringone was added only in inoculation medium increased percent of survived regenerative calli derived from freshlly isolated embryos was observed (Table 2). No clear correlation was found between the frequencies of transient expression and hygromycin-resistant and GUS postive plant regeneration. The highest rate of regeneration and transformation were obtained in conditions when freshly isolated immature embyos were used as a explants and acetosyringone was applied in inoculation medium (Table 2).

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Table 1. GUS expression in freshly isolated (FIIE) and precultivated (PCIE) immature embryos 3, 6 and 25 days after inoculation by *Agrobacterium* with acetosyringone added in inoculation and/or cocultivation media

Treatment	Explants	Acetosyringone 100 μM Inocul/Cocultiv	GUS expression, %		
			3 d	6 d	25 d
Control0-	FIIE	-/-	0	0	0
Control0	FIIE	+/+	0	0	0
Agro1	FIIE	+/+	68.4	66.7	11.1
Agro2	FIIE	+/-	66.7	70	66.7
Agro3	FIIE	-/-	62.5	80	64.7
Control2	PCIE	-/-	0	0	0
Agro4	PCIE	-/-	41.0	40.7	22.2
Agro5	PCIE	+/+	43.0	41.7	62.5

Table 2. Transformation of immature embryos and regeneration of hygromycin-resistant (Hyg^r) and GUS positive (GUS+) plants of wheat cv. Vesna

Treatment	No. of embryos inoculated	Calli (%)	Reg. calli %i	No. green spot/ callus	No. survived Calli, (%)	No. GUS+ Hyg^r plants	TE* %
Control0-	100	83	86.3	5.7	0	0	-
Control0	86	100	54.7	3.7	0	0	-
Agro1	150	81	24.0	3.0	19(16)	6	4.0
Agro2	125	85	34.9	6.4	32(30)	9	7.2
Agro3	225	46	20.2	4.9	15(14)	12	5.3
Control2	90	100	91.1	7.3	0	0	-
Agro4	100	92	2.2	1.0	3(3)	2	2.0
Agro5	130	70	0.1	1.6	3(2.3)	5	3.8

**TE-Transformation efficiency = (No. of survived plants/No. of inoculated embryos) x 100*

SONICATION ASSISTED *Agrobacterium*-MEDIATED TRANSFORMATION ENHANCES THE TRANSFORMATION EFFICIENCY IN FLAX (*Linum usitatissimum*)

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The use of *Agrobacterium tumefaciens* in transformation system based on its Ti plazmid has become the major tool for insertion of foreign genes into plant cells which then express desirable gene products. Using the co-cultivation of leaf disks with agrobacteria first transformed plants were recovered already in 1985 (Horsch *et al*., 1985). Since then many plant species have been successfully transformed. However, in certain species, to which belongs also flax (*Linum usitatissimum*), the transformation methods has reached rather slow progress and mostly has lead to unsatisfactory results. Trick and Finer (1997) reported, that in tissues tested, the Sonication assisted *Agrobacterium*-mediated transformation (SAAT) greatly enhanced the levels of transient expression of a GUS reporter gene. As tissues differ in their response to SAAT, each plant tissue needs its optimized protocol. We have used the SAAT to increase transformation efficiencies for flax hypocotyls.

Hypocotyls from about 10-day-old seedlings were cut into 8mm pieces and placed into liquid LB medium. The target tissue was subjected to pulses of ultrasound about 60 kHz and 35 kHz delivered by the apparatus. A and B, respectively. After sonication for 0-100 s the suspension was inoculated with *A. tumefaciens* strain LBA 4404 carrying binary vector pBIN m-gfp5-ER to a final OD_{600} 0,4 – 0,8 and co-cultivated 2 hours at 27 °C. Hypocotyls were blotted on a sterile filter paper and placed on MS medium for 2 day co-culture in the dark at 22 °C. A debacterization was provided using sterile water and solution of Claforan. Hypocotyls were then placed on MS medium with antibiotics. The sonication treatment results in thousands of microwound on and below the surface of the tissue. To reveal the degree of surface damage some hypocotyls were critical point-dried, gold-coated, and viewed with a Scanning Electron Microscope JOEL 6300. Monitoring of colonization and infection of hypocotyls was provided in real time using a Leica MZ 12 stereo dissecting microscope equipped with a fluorescence module consisting of 100 W mercury lamp and GFP excitation and emission filters integrated with CCD camera. Transient expression was observed and photographed immediately, and at 24, 36 and 48 hours after debacterization. Our results shows, that the treatment with ultrasound facilitates the uptake of plasmid DNA coding for GFP into cell of flax hypocotyls and its efficiency depends on the duration of treatment under frequency used. We have shown also that SAAT could be a promising method for enhancing the transformation efficiency of flax.

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GENETIC TRANSFORMATION OF *Linnum usitattisimum* USING DIFFERENT *Agrobacterium tumefaciens* STRAINS

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Flax (*Linum usitatissimum* L.) was one of the first crops domestificated by human and it is one of the most ancient and important world crops, widely applied in textile, paint and varnish, electrotechnical, rubber, tanning, pharmaceutical, food and other industries. Industrial use of flax plant material is in many cases affected by composition and quantity of lignin fibers, because after cellulose it is second most abundant organic compound. Lignin is a major cell wall polymer of plant vascular tissues, thus important for mechanical support and water transport in plants. It is a very heterogenous polymer therefore, there are lot of mechanisms how to modify quality as well as quantity of lignin. Way how we try to improve a quality of flax fibers was to modify the activity of cinnamyl alcohol dehydrogenase. Cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) is monolignol biosynthetic enzyme, catalyzes the final step of lignin subunit biosynthesis in higher plants.

For transformation we used cultivar Super. The explants were transformed with binary plasmid pAV04 containing CAD gene, driven by the CaMV 35S promoter, and NptII gene that provides resistance to kanamycin/geneticin Hypocotyl segments were transformed using modified method published by Mlynárová *et al.* (1994).

Because the effectivity of transformation is relatively low, we are trying to focus our recent work on improvement of transformation techniques and test different *Agrobacterium tumefaciens* strains (LBA 4404, GVA 3010) to obtain enough transgenic lines to analyse lignin content and the role of CAD in lignin modification and fiber quality.

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TRANSIENT EXPRESSION OF PROTEINS USING MODIFIED PLANT VIRAL VECTORS

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Plant viral vectors used for transient expression of proteins are an attractive alternative to conventional breeding and transgenic methodology. Transient expression is fast, flexible, unaffected by chromosomal positional effects and can be used in fully differentiated plant tissues (Fischer *et al*., 1999). Transient systems based on virus allow expression of foreign genes at higher levels in infected tissues than is normally the case in transformed plants (Yusibov *et al*., 1999).

We have modified potato virus X (PVX) based expression vector pGR106 by insertion of its modified coat protein bearing two human papillomavirus (HPV) epitopes. Epitope from E7 protein (44-60 aa) was fused either to N- or C- terminus of PVX CP and epitope from L2 protein (21-32aa) only to N- terminus. The coding sequences of these fusion proteins were obtained by PCR and cloned into the bacterial expression vector pMPM4. The constructs will be cloned also into the PVX based expression vector pGR106 and the level of fusion proteins expression in both systems will be determined. The plant expression system has the potential for use as vaccines.

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PLENARY SESSION IV: PLANT-PATHOGEN INTERACTION

UNDERSTANDING INTERACTIONS OF PLANTS AND VIRUS-LIKE AGENTS IS A KEY TO PATHOGEN CONTROL

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Currently we know about 1740 viruses, 26 ´*Candidatus* Phytoplasma´ species and 36 viroids infecting plants – virus-like agents which reduce the quality and yield of crops. Common feature of plant viruses, phytoplasmas and viroids is their intracellular parasitism, which makes their chemical control almost impossible. Virocidal chemical compounds e.g. synthetic uracyl analogues as ribavirin may be toxic to plants in higher concentrations, expensive for practical use and harmful to animals and human. Similarly ineffective is the injection of tetracycline antibiotics against phytoplasmas in infected trees – the symptoms reappear when treatment stops.

In consequence the current control of viruses, phytoplasmas and viroids is based on preventive measures and use of plants resistant to those pathogens and their vectors. Preventive measures are based on the use of certified virus-free or virus-tested seed and seedlings, assured by visual inspections and testing of propagative material by sensitive detection methods e.g. ELISA or PCR. Legislative measures as registrations of seed producers and issue of phytosanitary certificates for exported plants, act as important barriers against uncontrolled world-wide spread of harmful virus-like agents by international trade.

Plants resistant to virus-like agents may be selected from existing cultivars after their infection with viruses of economic importance. This is, however, long-term and laborious process and the resistance may be broken by mutation of the respective virus. Often such resistant cultivars are not available and resistance genes must be introduced from their wild relatives by traditional breeding methods or genetic engineering. Thus, maintaining of germplasm collections and protection of the diversity of cultural plants in areas of their origin is important for modern agriculture.

Virus-like pathogens display different mechanisms of replication in plants, depending on their genetic information. Viroids contain only RNA and phytoplasmas both RNA and DNA. In case of viruses, it can be single or double strand RNA or DNA. Nevertheless, some plant defense mechanisms against viruses are common.
Interactions of plants and viruses could be described as sensitivity, immunity, hypersensitivity and tolerance, terms expressing different levels of resistance. Immune plants can not be infected with the respective virus. Hypersensitive response is resulting in rapid death of virus-invaded cell, inhibition of cell-to-cell movement of the virus and invasion of the whole plant. Tolerance means replication of the virus and infection of plant with no apparent symptoms or acceptable level of yield and quality loss. Virus-tolerant cultivars represent source of infection for further spread of the virus in a crop. However, they are often accepted, in particular under conditions of strong infection pressure by abundant vectors, e.g. whiteflies or aphids in intensive cropping systems.

In plants the exploitation of resistance was often hampered by the fact, that it is only rarely based on a single dominant gene but more often on recessive multiple genes. Recent advances in plant genomics have brought extensive advances in localization of virus-resistance genes. Already the exploitation of markers of such genes in marker-

assisted selection or breeding enables rapid selection of resistant lines with multiple resistant genes. Genomes of viruses, viroids and phytoplasmas are relatively simple. In contrast to phytoplasma and viroids, the plant-virus interactions are understood much better. Many of them have been sequenced and the principal role of their "genes" in replication, movement in plants and spread in natural conditions was revealed.
Recently, studies on plant-virus interactions on cellular and molecular level revealed basic mechanism of plant defense against viruses - post transcriptional gene silencing (PTGS) and moreover mechanism of counter-defense of some viruses e.g. *Potyviridae,* against it. As result many plant virus-derived genes, both functional and truncated, expressed in numerous crops were proved to induce resistance. These genes include e.g. coat protein, insect transmission factors, movement protein and protease inhibitors. On the other hand demonstrations of recombination between a transgene and disabled virus raised many questions about the risk associated with the use of viral genes to develop resistance.

The main obstacle for broad use of transgenic crops expressing pathogen-derived genes or even plant-derived resistance genes is low acceptance of genetically modified crops in Europe. Nevertheless, the exploitation of transgenosis in the protection of plants against pathogens and insects as pests and virus vectors could bring reasonable reduction in energy costs, reduction of pesticide use and in consequence high quality and more healthy agricultural products.

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SCREENING OF Yr10 WHEAT (*Triticum aestivum* L.) YELLOW RUST (*Puccinia striiformis*) RESISTANCE GENE IN BREAD WHEAT CULTIVARS

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Yellow rust caused by *Puccinia striiformis* f.sp. tritici, is one of the most serious leaf diseases of wheat in the world.In the frame of a collaborative project our group working on development of stripe rust resistant genotypes using molecular techniques. Among the yellow rust resistance genes only the sequence of Yr10 (GenBank no: AF149112) was released. Polymerase Chain Reaction (PCR) was used to determine the presence of Yr10 in 7 different bread wheat varieties. Amplification products obtained with E1 primer pair (Forward 5' CTTGCTGGCGACCTGCTTA 3'; Reverse 5' TGTT TCGCTCCACGCTGACT 3') designed according to the sequence of the first exon and E2 primer pair (Forward 5' TGGTAGTAGAGTAATCGCAACA 3'; Reverse 5' TCTT CAGATTTGGAGGTAGG 3') designed according to the sequence of the second exon, in all varieties. Amplification products were obtained in 4 varieties (P.I.178383, Altay2000, Aytın98 and ES14) using E2A primer pair (Forward 5' TGGAAATGGAT AGGCGAAGG 3'; Reverse 5' AAATCAATGA AGCCGCAACC 3') designed according to the sequence of the second exon. According to the results of PCR with E2 forward and E2A reverse primers, it was shown that E2A reverse primer could not anneal to genomic DNA in 3 varieties (Harmankaya99, İzgi01 and Sönmez2001). 1311 bp PCR products of 4 varieties obtained using E2 forward and E2A reverse primers and 754 bp PCR products of 7 varieties obtained using E1 primer pair were subjected to sequence analysis. Examination of the sequencing results and the calculation of the similarity scores were carried out using ClustalW "multiple sequence alignment program". Jalview "a multiple alignment editor" was used to visualize nucleotide sequence alignments. Sequence analysis showed that the varieties which is most similar to the first exon of Yr10 are Altay2000 and P.I.178383 and the variety which is most similar to the second exon of Yr10 is P.I.178383. It was observed that 3 varieties (Harmankaya99, İzgi01 and Sönmez2001) are the least similar to the first exon of Yr10. The results obtained from this work indicate that (1) Yr10 gene is present in all of these varieties, (2) the divergence between the varieties is arised from the variations in the second exon and (3) the first exon is more conserved than the second exon. This is the first study carried out to examine the variations of Yr10 using PCR and sequence analysis. The results of this work will contribute to determine the divergence between resistant and susceptible varieties and will be helpful to breeding applications.

GENETIC COMPARISON OF TWO COMOVIRUSES INFECTING *Brassicas*

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An isometric comovirus was isolated from a sample of Chinese cabbage (*Brassica pekinensis*) plant with mosaic symptoms collected about 15 years ago in Moscow (Russia). This virus reacted weakly with Radish mosaic comovirus (RaMV) antiserum (Špak and Kubelková, 2000) and until now it has been supposed to be an isolate of RaMV.

Comoviruses are characterized by their bipartite, ssRNA genomes, expressed as polyproteins, one from RNA-1 (ca. 5900 nt), and two (inframe, overlapping and sharing a common termination codon) from RNA-2 (ca. 3600 nt) (Goldbach and Wellink, 1996). The RNA-1-encoded polyprotein yields the RNA-dependent RNA polymerase (RdRp), a helicase and a protease, as well as a protease cofactor and the genome-linked viral protein (VPg), while the RNA-2-encoded polyproteins yield the movement protein, large capsid protein (CP-L) and small capsid protein (CP-S). Each of RNA is 3´-polyadenylated, is linked to a 5´-VPg and is individually encapsidated by 60 copies each of CP-L and CP-S proteins to form icosahedral particles of 28 nm in diameter.

Till PCR amplification experiments revealed differences in ability of the viral cDNA of the Russian isolate to hybridise with RaMV-specific primers. Subsequent stepwise specific amplification and sequencing was used to obtain about 2700 nt long 3´-terminal part of RNA1 and the complete nucleotide sequences of the capsid protein genes of the Russian isolate. Their CP-L and CP-S genes have about 70.4% and 66.7% nucleotide sequence identity and 78,7% and 63,5% aminoacid identity with RaMV, respectively. There is also only 72% aminoacid identity in RdRp protein between the Russian isolate and the RaMV1 isolate.

Furthermore, BLAST search showed 100% identity of the received RdRp sequence with 55% sequence coverage of RdRp deposited in the GenBank as a tentative comovirus and under the name *Turnip ringspot virus*.

The aa identity level of the RdRp below 75% with RaMV fulfil the criteria for discrimination of the Russian isolate as a new comovirus species. Although the identity of the CP-L is above, but very tightly, to this limit, we propose to discriminate the Russian isolate as a new species of the genus *Comovirus* and give it the name *Turnip ringspot virus* (TuRV).

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GENETIC VARIABILITY OF OAT CROWN RUST ISOLATES

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Oat crown rust, caused by basidiomycete fungus *Puccinia coronata* Cda f. sp. *avenae* Eriks. is a major disease of oats in many regions of the world including the Czech Republic. Rust populations can be characterized using both plant pathologic and molecular approaches. Studies of physiologic specialization, based on Flor's gene-for-gene hypothesis, proved that *P. coronata* f. sp. *avenae* populations are extremely diverse in virulence (Chong and Zegeye, 2004). Knowledge of genetic background of the pathogen populations on DNA level is not as well studied as in case of another cereal rust species. Objectives of the study were to (i) assess genetic variability of oat crown rust isolates by means of Amplified Fragment Length Polymorphism (AFLP) analysis; (ii) analyse if the correlation between genetic variability, as detected by AFLP, and physiologic specialization of the oat crown rust isolates occurs.

Forty monopustule isolates of oat crown rust originated in various European countries and Israel, collected in the period from 1999 to 2005, were included (Table 1). The analyses of physiologic specialization were carried out according to Klenová and Šebesta (2006) using 16 single gene oat lines (*Avena sativa*) with seedling resistance to *P. coronata* f. sp. *avenae*. The nomenclature system proposed by Chong *et al*. (2000) was used for pathotype designation. Twenty-nine different pathotypes were identified among the isolates possessing 0-11 virulence genes. Majority of pathotypes was identified in individual cases, four pathotypes were recorded twice, the pathotype BLBG occurred five times.

AFLP analysis (Vos *et al*., 1995) was carried out according to the AFLP™ Plant Mapping protocol (Applied Biosystems). Twelve primer pairs were used for selective amplification. For each primer pair, 13-58 DNA fragments, ranging in size from 50 to 495 bp were detected. A total of 501 AFLP polymorphic fragments were scored with all the isolates; it corresponds to an average of 41 polymorphic bands per primer combination. UPGMA clustering analysis distinguished the isolates into 5 clusters with different virulence/avirulence gene distribution based on level of linkage distance 0.27 (Figure 1); two clusters are formed by single isolates. Three robust clusters differed with number of virulence genes, the first two clusters comprise isolates with up to four virulence genes, and the third cluster comprises mostly isolates possessing from 4 to 11 virulence genes. The structure of the clusters also indicates that the urediospores of oat crown rust often migrate between the Czech Republic, Austria and Serbia (see the second cluster) or between Israel, Serbia and Estonia (see the third cluster). Most of the isolates originated in the Czech Republic share one cluster, which indicate either prevailing asexual reproduction or development of subpopulations surviving at the local areas over the years. The comparison of UPGMA clustering analyses for the physiologic specialization and AFLP data set using indicated a low, but highly confirmative correlation.

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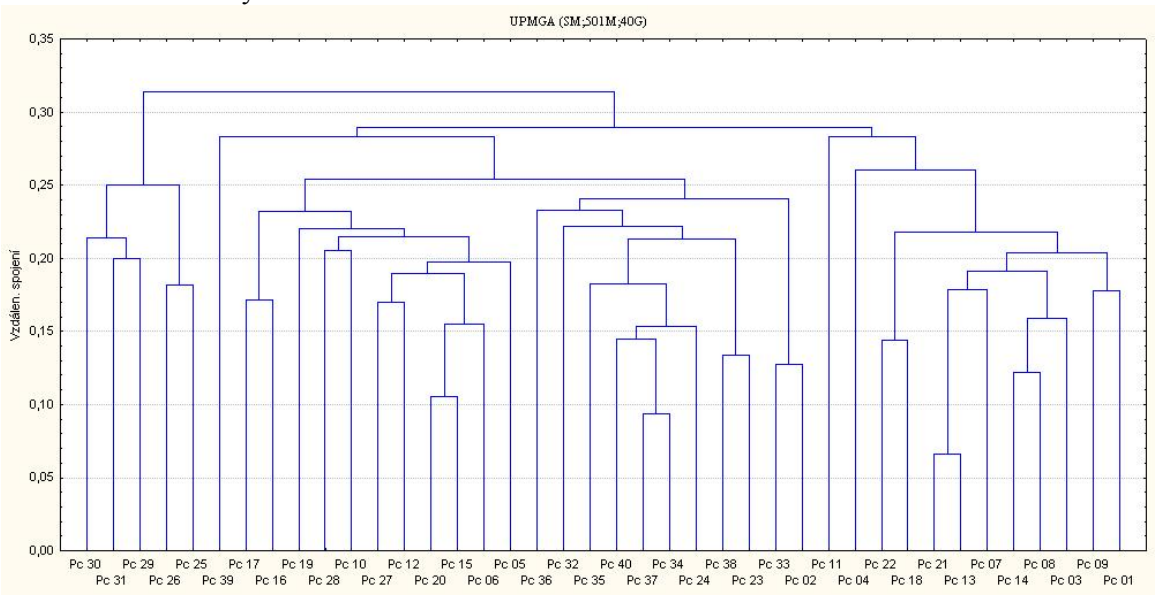
Table 1. Pathotype designation of *Puccinia coronata* f. sp. *avenae* isolates originated in different European countries and Israel collected from 1999 to 2005

Pca isolate designation	Origin	Year of sampling	Virulence pattern	*Pca* isolate designation	Origin	Year of sampling	Virulence pattern
Pc 1	ISR	2005	SGBB (4)	Pc 21	EST	2004	SBLM (6)
Pc 2	ISR	2000	QLBG (4)	Pc 22	ISR	2004	TSFM (11)
Pc 3	CZ	2005	SBLR (7)	Pc 23	YU	2000	BBBB (0)
Pc 4	SWE	1999	LBBG (2)	Pc 24	CZ	2004	SLLM (7)
Pc 5	CZ	2004	SBNM (7)	Pc 25	CZ	2004	QBBG (3)
Pc 6	YU	2000	GBBB (1)	Pc 26	YU	2004	BLBG (2)
Pc 7	YU	2004	SBLC (5)	Pc 27	CZ	2005	BLBG (2)
Pc 8	SWE	2000	LBBC (2)	Pc 28	CZ	2005	BLBB (1)
Pc 9	YU	2004	TBNM (7)	Pc 29	CZ	2004	BBBC (1)
Pc 10	SWE	1999	GLBB (2)	Pc 30	EST	2004	SBLB (4)
Pc 11	CZ	2005	LBBC (2)	Pc 31	ISR	2004	QBBL (3)
Pc 12	CZ	2004	BBBB (0)	Pc 32	AUT	2000	LMBB (3)
Pc 13	EST	2004	SBLM (6)	Pc 33	AUT	2000	BLBC (2)
Pc 14	EST	2004	SBBC (4)	Pc 34	AUT	2000	BLBG (2)
Pc 15	CZ	2004	BLBG (2)	Pc 35	YU	2000	QLBM (5)
Pc 16	CZ	2005	BLBD (2)	Pc 36	AUT	2000	BLBG (2)
Pc 17	HU	2005	GLBD (3)	Pc 37	AUT	2000	BLBG (2)
Pc 18	ISR	2004	TSFM(11)	Pc 38	EST	2000	GBBG (2)
Pc 19	CZ	2005	QQBB (4)	Pc 39	SWE	1999	BLBB (1)
Pc 20	CZ	2004	BLBG (2)	Pc 40	CZ	2000	GLBG (3)

Pca: Puccinia coronata f. sp. avenae
Pc 1 – Pc 40: designation of Puccinia coronata f. sp. avenae isolates
***AUT**: Austria, **CZ**: Czech Republic, **EST**: Estonia, **HU**: Hungary, **ISR**: Israel, **SWE**: Sweden, **YU**:Serbia*
Four letter code - nomenclature system for Puccinia coronata f. sp. avenae (Chong et al., 2000); numbers in brackets represent number of virulence genes in the isolate

Figure 1. Dendrogram of genetic diversity of *Puccinia coronata* f. sp. *avenae* as detected by AFLP analysis



Pc 1 – Pc 40: designation of Puccinia coronata f. sp. avenae isolates

PRELIMINARY RESULTS OF *IN VITRO* THERMOTHERAPY OF APPLE CULTIVARS IDARED AND SAMPION

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Apple cultivars ´Idared´ and ´Sampion´ were selected for application of *in vitro* thermotherapy at 39 ± 0.5 °C. The presence of viruses in selected initial trees was detected by ELISA and RT-PCR testing before the beginning of thermotherapy. Cultivars ´Idared´ and ´Sampion´ were both infected with *Apple chlorotic leaf spot virus* (ACLSV) and with *Apple stem pitting virus* (ASPV). Cultivar ´Sampion´ was moreover infected with *Apple stem grooving virus* (ASGV). Two apple cultivars were successfully multiplied in *in vitro* cultures. Multiplication coefficient was 2.9 ± 0.2 for ´Sampion´ and 2.8 ± 0.2 for ´Idared´ after four weeks of cultivation on MS medium with 1.5 $mg.l^{-1}$ BAP. After the end of thermotherapy, one variant of apple cultivar ´Idared´ was free of all tested viruses after transfer to *ex vitro* conditions and repeated RT - PCR testing. Achieved results are preliminary. Other sanitation and testing of apple cultivars used in the project will be carried out in the following years.

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DIFFERENTIAL GENE EXPRESSION IN APPLE TREATED WITH RESISTANCE-INDUCING FACTORS

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The aim of the study was to identify genes whose expression is affected by treatment with factors inducing systemic resistance mechanisms in apple (*Malus domestica* Borkh.). For isolating genes differentially expressed after treatment with resistance inducers, cDNA-AFLP method was applied. Young apple trees cv. 'Ligol' were sprayed with extracts from mistletoe (*Viscum album*) and ivy (*Hedera helix*), solution of "Messenger" (harpin protein) and bacteria *Pseudomonas chlororaphis* suspension, while control plants were sprayed with water. Leaves for molecular analyses were collected from treated and control plants 2, 7 and 14 days after treatment. Analysis was performed as described by Bachem *et al*. (1996). Products of selective amplification were separated on 6% denaturing polyacrylamide gels and silver stained. Amplification products showing differential expression pattern in control and treated plants were re-amplified from polyacrylamide gel, cloned into pGEM-T vector and sequenced. Sequences of cDNA fragments were subjected to BLAST analysis. Until now, 26 cDNA fragments shoving differential expression (either up- or down-regulated) were identified and 16 of them were successfully cloned and sequenced. In the BLAST analysis, the homology with genes encoding known proteins was found for 6 of cloned cDNA fragments. Confirmation of differential expression of identified genes in response to treatment with resistance-inducing factors will be done using real-time RT-PCR technique.

The effect of applied resistance-inducing factors on expression pattern of defence gene analogs from apple was also studied. The analysis was performed using semi-quantitative RT-PCR technique. The specific primers used the in reactions were designned basing on sequences available in databases. The obtained PCR products were cloned into plasmid vectors and sequenced to confirm their identity with original sequences. Up to now, expression pattern of 3 genes were analysed: β-1,3-glucanase, polyphenoloxidase and phenylalanine ammonia lyase. Actin and 18S rRNA genes, showing constitutive expression, were used as a reference. The analysis did not show changes in expression pattern of analysed genes between control apple plants and plants treated with applied factors.

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PARTIAL MOLECULAR CHARACTERIZATION OF ONION YELLOW DWARF VIRUS ISOLATES INFECTING GARLIC

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Onion yellow dwarf virus (OYDV, *Potyvirus*) is a member of *Potyviridae* family of plant viruses. This virus causes various degrees of stunting, irregular yellow striping, and downward leaf curling of an onion, garlic, and the other *Allium* plants. Recently it has been reported worldwide, often in mixed infection with *Leek yellow stripe virus* (LYSV, *Potyvirus*), *Garlic common latent virus* (GarCLV, *Carlavirus*), *Shallot latent virus* (SLV, *Carlavirus*), and *Garlic mite-borne filamentous virus* (GarMbFV, *Allexivirus*). All of them are caused of an important reduction of yield and quality of production. *Onion yellow dwarf virus* is transmitted in non-persistent manner by the aphids. Transmission through seed seems improbable. Exchange and import of OYDV host species mainly vegetatively propagated garlic seed material constitutes the possible way of spreading.

The collection of OYDV isolates has been provided from different plant material in the Czech Republic in period 2003-2006. Thirteen OYDV isolates representing field onion (1), field garlic (4), imported Chinese garlic from hypermarket (2), and garlic gene resources (6) were included in the study. In all cases, the presence of viruses was confirmed using DAS-ELISA (Bioreba AG).

The two overlapping fragments covering 5´(Nter)NIb-CP-UTR3´ region in total length cca. 1020 bp were prepared using RT-PCR. The first amplicon was obtained by OYD-UP/OYD-DW primer pair, the second amplicon was obtained using newly designed OCP2 and potyvirus universal P9502 primers. The each amplicon was cloned into pGEM-T plasmid (Promega) and sequenced using BIG DYE v.1.1 sequence terminator kit and ABI PRISM 3130 sequencer (both Applied Biosystems).

The obtained sequences were compared with GenBank available OYDV sequences and aligned using ClustalW. The variable region of CP gene was used for the construction of the phylogenetic tree.

Phylogenetic analysis discriminated isolates into seven clusters. The all originally field Czech isolates coming from onion and garlic, except one, clusterized with the known European and Israel OYDV isolates. Newly sequenced OYDV isolates obtained from imported Chinese garlic and from garlic gene resources formed group with the other common Asia, Japan and Chinese, OYDV garlic isolates, what reflects their geographical origin. The two exceptions from this situation were noted. The Japan isolate ´Gams´ clusterized with European isolates, and the Czech field isolate DVE7CZ is close relative to isolates coming from China. This distribution indicates the possible way of OYDV isolate/gene flow among locations through commercial garlic and infected gene resources accessions. This fact of isolate and/or possible pathotype mixing represents the real phytosanitary risk.

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PROGRESS IN RADISH MOSAIC VIRUS SEQUENCING

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The genus *Comovirus* contains fifteen definitive virus species. One of them is *Radish mosaic virus* (RaMV) which differs in the host range plants: It is the only one comovirus infecting *Brassicaceae,* till now.

The genome of typical comovirus is made of two genomic ssRNAs with a 5´-bound polypeptide and a 3´-poly(A). The smaller segment is 3300 to 3800 nt, the larger one is 5900-7200 nt. Both segments are translated into polyproteins and processed with viral protease up to ten proteins. The Helicase-VPg-Protease-Polymerase and Movement protein-Large capsid protein-Small capsid protein arrangements are used for these two polyproteins. As in other picorna-like viruses, the polymerase protein is the most conservative protein with 45-62 % of aminoacid identity between individual species. Unfortunately, outside of the core part with Ia-VIII polymerase motifs, only extremely few conserved motifs are present on this gene. With one exception, there does not occur any conserved stretch longer than 3 aa (Petrzik *et al*., 2005). This fact hampers searching sites suitable for specific amplification primers. Two nonspecific primers were used in low stringency annealing conditions on the RNA template prepared from purified virions, therefore.

Three short nucleotide segments resembling comovirus genome (two in the polymerase gene and one in the CP-L gene) were the starting point for the walking-primer sequencing approach. Specific reverse primers were designed in the read sequence, while the upstream primers were often highly degenerative and were designed in sites of the highest conservancy of the genes. The first 1000 nucleotides of the smaller genomic segment were determined by 5´-RACE method. Another closely related comovirus was detected during screening of our collection of isolates. This virus (*Turnip ringspot virus*) was found in mixed infection with RaMV. Its nucleotide sequence was about 70% identical with that of RaMV, and complicates specific amplification of only one virus.

We revealed, that the polyprotein-coding reading frame of RaMV RNA2 segment is terminated with TAG followed with 278 nt long noncoding region. The alignment with previously sequenced viruses resulted in the identification of the cleavage sites: The CP-L protein is separated from the MP protein in cleavage between Q/T and is 375 aminoacid long. The CP-L/CP-S cleavage site is Q/G. The CP-S gene encodes for protein of 245 aa.

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MOLECULAR CHARACTERIZATION OF CZECH PEA SEED BORNE MOSAIC VIRUS ISOLATES

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Pea seed borne mosaic virus (PSbMV) is a member of *Potyviridae* family of plant viruses. This virus causes various degrees of stunting, downward rolling of leaflets, and the transient clearing and swelling of leave veins of the most cultivars of *Pisum sativum*. It was discovered in 1966 in Czechoslovakia, but in recent it has been reported worldwide. PSbMV is transmitted in non-persistent manner by the aphids and downward because seeds, so the exchange/import of pea material constitutes the possible way of spreading.

The survey of PSbMV occurrence has been provided in the Czech Republic in period 2003-2005. The pea plants showing the typical symptoms were collected and the viruses were mechanically transmitted to experimental pea plants. In all cases, the presence of viruses was confirmed using DAS-ELISA (LOEWE Biochemica GmbH).

The eleven isolates; originating from three different localities, Smržice (4), Šumperk (4) and Troubsko (1), and ´Raman´ seed material (2), respectively; were chosen for detailed molecular analysis. The fragments covering 5´(Nter)NIb-CP-UTR3´ region in length 1108 bp or 1115bp were prepared using RT-PCR. The two overlapping amplicons, were obtained with the three different primer pair combinations, the first using potyvirus universal primers P9502/CPUP, and the second using newly designed primers PSB8812/PSB9440 or PSB8800/PSB9440. The amplicons were cloned into pGEM-T plasmid (Promega) and sequenced using BIG DYE v.1.1 sequence terminator kit and ABI PRISM 3130 sequencer (both Applied Biosystems).

The obtained sequences were compared with GenBank available PSbMV isolates sequences and aligned using ClustalW. The variable region of CP in length 412 bp allowing comparison of the largest number of isolates was used for the construction of the phylogenetic tree.

Phylogenetic analysis discriminated isolates into four groups. The all Czech isolates, except one, clusterized in Group 1 together with the known European PSbMV isolates coming from Denmark, Germany, United Kingdom, and the isolates from North America and Pakistan. Only one Czech isolate, PSB58CZ, clusterized in different branch, Group 4, together with isolates coming from USA, Australia and Egypt.

Distribution of the isolates into the four groups corresponds with the dividing PSbMV isolates into pathotypes according to their infection profile on *Pisum sativum* lines. All characterised type isolates of pathotypes P-1, P-2, P-3 and P-4 are the members of formed branches, i.e. groups, so the classification of our isolates into pathotypes P-1 and P-4 could be deduced. Next biological testing of Czech PSbMV isolates on different pea cultivars to validate this diversification is in progress.

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POSTERS

CELLULAR PHONE SIGNALS ON BACTERIA GROWTH AND NUCLEIC ACIDS (DNA & RNA) DEGRADATION

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Mobile or cellular telephones are long range, portable electronic devices for personal communications over long distances. Mobile phones are very similar to those cordless telephones although cordless ones generally operate only within a limited range of a base station. Today's mobile telephones support many additional services such as short message service (SMS), text messaging, email, packet switching for access to the internet and multimedia messaging service (MMS) for sending and receiving pictures and video. The use of cell phones has been dramatically increased, for instance, the number of mobile telephones has surpassed the number of people in England and Turkey. It is estimated that there would be over four hundred million mobile telephone users in China by 2008. There are several scientific evidences and public contentment about the mobile telephone uses and health problems including the brain cancers and other cancers. In the present study we intended to investigate the effects of cellular telephone signals on bacteria growth and extracted nucleic acids (DNA and RNA) degradation. The study material consisted of *Escherichia coli* carrying plasmid pUCR-*TaqPol*, plant genomic DNAs, RNAs (cotton and pepper), bacterial genomic DNA and plasmid DNA (*E. coli*, pUCR-*TaqPol*). Plant genomic DNAs and total RNAs were extracted according the protocol of Karaca *et al*. (2005) and Karaca *et al*. (2004), respectively. Bacterial genomic DNA and plasmid DNA extractions were performed according to Sambrook and Russell (2001). The study also utilized four mobile telephones which are most commonly used in the world. Bacteria cultures received continuing cellular telephone calls (signals) and SMS for various time intervals. The concentration of the bacteria culture was conducted using a spectrophotometer and the standard bacteria plate counting was utilized to determine the number of colony. The effect of the cellular telephone signals on nucleic acid degradation was assessed using the standard agarose gel electrophoresis (AGE) and ethidium bromide (EB) staining. Staining densities of the nucleic acids on gels were measured using Photoshop software. All the experiments were conducted using completely randomized design with four replications. Results of the present study clearly indicated that bacteria in cultures received continuous mobile telephone signals for 12 hours showed higher number of colony and higher spectrophotometer readings at OD_{600}. On the other hand the plate counting and the spectrophotometer readings of the bacteria in cultures that received one SMS at 1 hour and 10 minute time interval did not show any differences in comparison to controls (Table 1). Results indicated that continuous mobile telephone signals for 12, 24 and 36 hours (Figure 2) did not affect the nucleic acid integrity. Overall studies indicated that mobile telephone signals do not degrade nucleic acids *in vitro* conditions but do stimulate and enhance microorganism growth.

Table 1. Spectrophotometer readings at A_{600} nm of the bacteria cultures which received mobile telephone signals

	Replications			
	1.	2.	3.	4.
Control	0.717	0.719	0.718	0.718
SMS at 1 h intervals	0.718	0.717	0.719	0.718
SMS at 10 min intervals	0.719	0.718	0.718	0.719
Continues calls	0.723	0.728	0.731	0.725

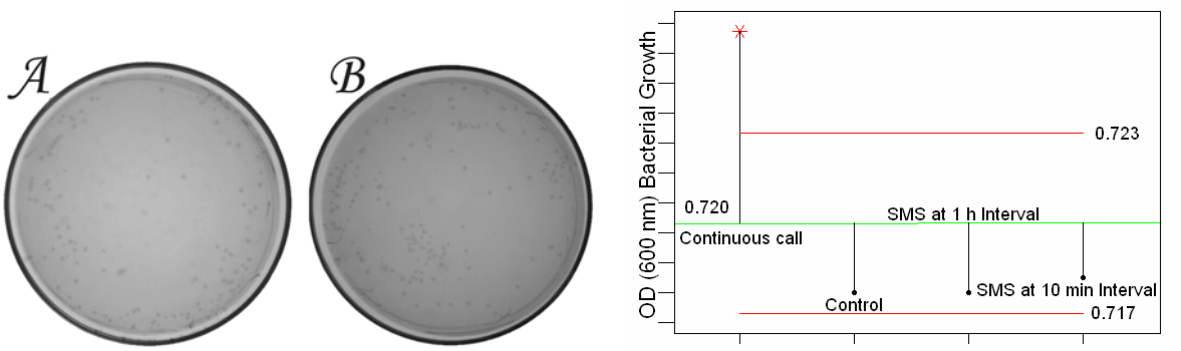

Figure 1. Plate counting of control (A) and continuing mobile telephone calls. The graph above show the significant effect of continues call signals on bacteria growth

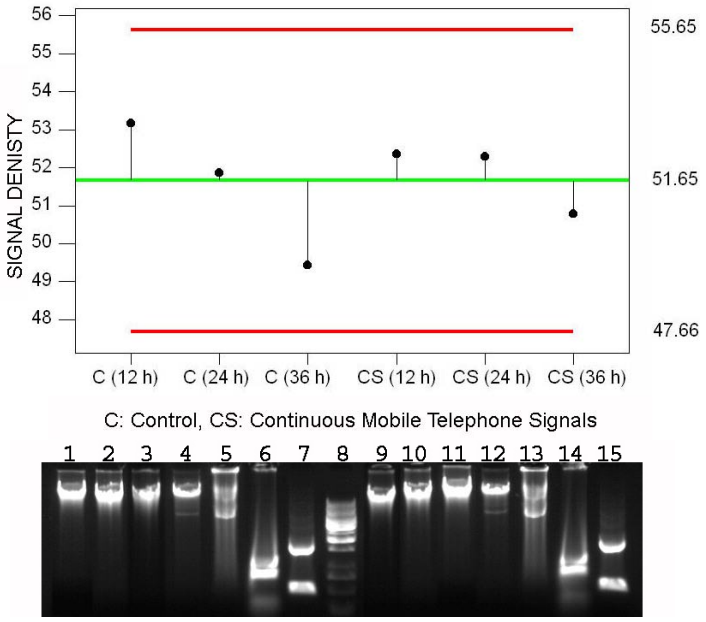

Figure 2. Ethidum bromide signal densities to measure effect of mobile telephone signals on nucleic acids (above) and agarose gel electrophoresis of cotton (lane **1** and **9**), pepper (lane **2** and **10**), eggplant (lane **3** and **11**), *E. coli* (lane **4** and **12**) genomic DNAs. Lane **5** and **13** show plasmid and genomic DNA, Lane **6** and **14** are cotton RNAs. Lane **8** is the DNA ladder. Lane **7** and **15** are the polymerase chain reaction (PCR) products amplified from cotton DNAs. Samples in lanes **1** to **7** received continues mobile phone calls for 36 hours. Lanes **9** to **15** are the control samples which did not receive call signals (below).

THE IDENTIFICATION AND CONTROL OF BACTERIAL CONTAMINANTS IN RED RASPBERRY AND BLACKBERRY CULTURES

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Bacterial contamination is a troublesome issue in tissue culture. The microorganisms gain access to the culture vessels due to insufficient surface sterilization of the initial explants or colonization of internal tissues, or as a result of manipulation in the laboratory. *In vitro* explants could contain bacteria arising at different stages of the culture. Endophytic bacteria can colonize plant tissues without obvious symptoms, and may reduce the multiplication rates and rooting capability, or even kill plant tissues. We attempted to identify bacteria isolated from *in vitro* cultures of ten raspberry and two blackberry cultivars, and to eliminate them from the tissues. Using standard bacteriological tests for bacterial classification, we identified 36 bacterial isolates. Most of the contaminants were Gram-negative, rod-shaped, non-spore forming *Pseudomonas* species, but we also identified *Enterobacteria*, *Staphylococcus*, *Streptococcus*, *Methylobacterium*, *Bacillus*, *Xanthomonas*, *Micrococcus*, *Lactobacillus* and *Alcaligenes*. Three isolates were classified as pink yeast (probably *Rhodotorula* or *Torulopsis*) and two isolates were described as pleomorphic forms. The best results in eliminating contaminants from tissues proved to be the addition of cefotaxim to the medium at a concentration of 250 $mg.l^{-1}$, or gentamycin at 50 $mg.l^{-1}$, or 0.2% Plant Preservative Mixture® (PPM).

MICROMYCETES OF GENUS *Fusaruium* ON BARLEY

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In 2005-2006, after maize growing as a forecrop in locality in the Czech Republic (Ivanovice na Hané), the level of infection in ears, determine of *Fusarium* species and analyze for content of mycotoxins were made. Three different methods of protection were used: 1) susceptible and resistant varieties of barley to attack of *Fusarium* species, 2) varieties of maize as a forecrop transgenic Bt-maize (enrichment of delta endotoxin from soil bacterium *Bacillus thuringiensis*) and its nontransgenic izolinie and 3) the fungicides protection and control variant were used. The most frequent micromycetes were *Fusarium graminearum* (54.5 %), *F.poae* (24.3 %), *F.tricinctum* (13.7 %), *F.avenaceum* (6.3 %) and *F.culmorum* (1.6 %). The content of mycotoxins Deoxynivalenol and Nivalenol in all variants of barley grain was detected. The close positive correlation between the isolation species and content of mycotoxins was demonstrated.

Acknowledgement

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POTENTIALLY TOXIGENIC MICROMYCETES ON TRANSGENIC *BT*-MAIZE AND NONTRANSGENIC HYBRIDS OF MAIZE

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The potentially toxigenic species of genera *Fusarium, Aspergillus,* and *Penicillium* are widespread and common fungi infecting maize and causing a wide range of diseases. These species cause reduction in grain yield and quality and their secondary metabolites – mycotoxins, which can occur in infected grains, have adverse effects on humans and livestock. The most frequent and known mycotoxins are deoxynivalenol, fumonisins, zearalenon, HT-2 and T-2 toxin, aflatoxins, ochratoxin and patulin.

The toxigenic species enter into maize through different routes. The very often route is damage of plants created by insects, especially by European corn borer (ECB) which is the major pest of maize in the Czech Republic. This damage of plants and their ears is often the initial infection site for toxigenic species.

Bt-transformed maize can reduce the occurrence of toxigenic species and minimize the risk of mycotoxins contamination in maize. This transgenic Bt-maize contains the gene from soil bacterium (*Bacillus thuringiensis*) express the toxic Cry 1 Ab protein which protects the maize against European corn borer.

During the years 2002-2005 different samples of corn (preharvest, harvest and postharvest) from different localities of the Czech Republic (Ivanovice na Hané, Praha-Ruzyně, Troubsko and Potěhy) were collected. The main aim of this study was to analyse the spectrum of toxigenic species occuring in different samples of maize (preharvest ears, harvest and postharvest grains). In total, 47 potentially toxigenic species were isolated. The highest number of isolated species was in the years 2002, 2005, and from the locality Praha-Ruzyně. The most frequent species were *Fusarium subglutinans, F. verticillioides, F. proliferatum, F. poae, F. sporotrichioides, Aspergillus fumigatus, A. versicolor, Penicillium crustosum, P. chrysogenum* and *P. expansum*.

The efficacy of Bt-maize was showed in lower occurrence of potentially toxigenic species.

Acknowledgement

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WHEAT LEAF SPOT DISEASES IN THE CZECH REPUBLIC IN THE PAST FEW YEARS

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The most important causal agents of wheat leaf spot diseases are *Phaeosphaeria nodorum* (anam. *Stagonospora nodorum*)*, Mycosphaerella graminicola* (anam. *Septoria tritici*) and *Pyrenophora tritici-repentis* (anam. *Drechslera tritici-repentis*). The next species are *Alternaria alternata, Cladosporium cladosporioides* and *Epicoccum nigrum.* In the past few years the occurrence of these pathogens has been changing in the Czech Republic. In the past *P. nodorum* was the most important wheat leaf spot pathogen in our conditions but nowadays *P. tritici-repentis* has been spreading to our country and to other European countries as well because of changes in cultural practices.

P. tritici-repentis (PTR) causes wheat leaf spot disease known as "tan spot or "yellow spot". This homothallic ascomycete has been reported on many grass species from different parts of the world. *PTR* isolates are separated into 8 races according to their virulence/avirulence combination to cultivars/lines used as a differential set. So far four host-specific toxins (Ptr Tox A, B, C, D) produced by *PTR* have been characterized. The toxins are responsible for induction of chlorosis, necrosis or both types of symptoms. Variability of *PTR* population was studied in different parts of the world and generally races 1 and 2 seem to be the most common.

The aim of this study was to summarize results dealing with the incidence of wheat leaf spot pathogens in the last five years in the Czech Republic and to give preliminary information about race spectrum of *PTR* in our country.

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SYSTEMIC RESPONSE OF TRANSGENIC *IPT* AND CONTROL TOBACCO TO *POTATO VIRUS Y* INFECTION

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We studied effects of *Potato virus* Y^{NTN} (PVY) infection in control (*Nicotiana tabacum* L., cv. Petit Havana SR1; C) and in P*ssu-ipt* transgenic (T) tobacco overproducing endogenous cytokinins (CK). Both types of plants were grown as rooted (C, T) or grafted onto control rootstock (C/C, T/C) under greenhouse conditions (Synková *et al*., 2006). The plants were mechanically inoculated under the same conditions. The first symptoms of PVY infection were observed in C and C/C after ca 7-10 days after the inoculation in systemically infected leaves. Later (ca 15 days after the inoculation) most of T plants showed also visible symptoms, while T/C sustained the infection without any symptoms. In our present experiment, we focused on histochemical methods to study the effects of PVY in systemically infected leaves. Fresh leaf hand-cut sections were stained for lignin by phloroglucinol method, for peroxidase and NADP-malic enzyme (ME) activity, and UV or blue light excitation was used for a detection of phenolic compounds autofluorescence (Schnablová *et al.*, 2006).

In healthy C and C/C, only xylem and/or phloem vessels were visible in blue or yellow autofluorescence or stained by phloroglucinol. Activity of peroxidases was also localized around the vessels or in epidermal cells, while ME activity was detected in various clusters of cells along the leaf section. PVY infection caused enhanced autofluorescence of phenolics, and increased activities of peroxidases and ME along the whole leaf section. Higher ME activity was found in cells adjacent to necrotic sites that appeared as symtoms of the infection. Phloroglucinol staining was restricted to xylem vessels and did not show any difference from healthy plants.

Contrary to controls, transgenic plants (particularly T/C) showed higher autofluorescence of phenolics throughout the leaf section in the absence of the virus. Higher activities of peroxidases and ME were also found in healthy transgenic tissue. Contrary to controls, phloroglucinol stained cells were not restricted only to the vessels but they were localized adjacent to necrotic cells occurring in transgenic tissue in the absence of PVY. The infection did not change this picture, although the accumulation of the virus (but lower compared with C) was confirmed in T and T/C.

We conclude that the spontaneous formation of necrotic lesions in the absence of the virus on leaves of transgenic tobacco during plant ontogeny could be the reason for its higher tolerance to PVY. It might be caused by local CK overproduction that activates cell death and it is accompanied by the induction of multiple defense mechanisms and the induction of enhanced resistance, similar to systemic aquired resistance. This has been suggested also for various transgenes with hypersensitive like lesions (Mittler and Rizhsky, 2000).

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PEPTIDES WITH ANTIMICROBIAL ACTIVITY ISOLATED FROM PLANTS

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Plants as other natural organisms in any phase of development can be contaminated by a wide range of microorganisms, including viruses, bacteria, fungi, nematodes and insects. In order to survive, plants must possess a defense system to block the entry of these invaders. Several molecules in plants have been reported to contribute to plant defense activity:(1) proteins or peptides, such as lectins, ribosomal inactivating proteins, chitinases, proteases, defensins, peroxidases, ubiquitin-like peptides, ribonucleases, arginine- and glutamate-rich peptides and some unclassified proteins, (2) organic compounds, classified into phytoanticipins and phytoalexins, which include phenols and phenolytic glycosides, unsaturated lactones, sulphur compounds, saponins and dienes, and (3) active nitrogen and oxygen species, such as reactive nitrogen oxide species and hydrogen peroxide.

During the past 15 years, a large number of antimicrobial proteins have been identified in different plants. Antimicrobial peptides constitute a heterogenous class of low molecular mass proteins, which are recognized as important components of defense system. These peptides exhibit broad-spectrum activity against a wide range of microorganisms including G^+ and G^- bacteria, protozoa, yeast, fungi and viruses. A few peptides have also been found to be cytotoxic to sperm and tumour cells. Consequently antimicrobial peptides have been found to be excellent candidates for developing of novel antimicrobial agents and a few of these peptides show antimicrobial activity against pathogens causing sexually transmitted infection, including HIV/HSV.

The aim of this study is isolation and characterization of substances with antimicrobial and antifungal activity from plants. Several families of plant antimicrobial peptides have been isolated from different organs (seeds, leaves and flowers) and intercellular washing fluids. Antimicrobial peptides were isolated by precipitation of fractions and separated by RP-HPLC and then characterized by UV-VIS spectroscopy, SDS-PAGE electrophoresis and mass spectrometry. Antimicrobial and antifungal activity was tested using bacterial strains G^- - *Escherichia coli, Pseudomonas aeruginosa, Agrobacterium rhizogenes*; G^+ - *Micrococcus luteus, Staphylococcus aureus, Bacillus subtilis, Bacillus megatherium, Bacillus cereus, Bacillus pumillus, Arthrobacter ureafaciens* and fungi *Fusarium culmorum, Cladosporium herbarum, Aspergillus terreus, Trichoderma virens, Aspergillus oryzae, Aureobasidium pollulans, Geotrichum candidum* a *Alternaria* sp. The screening method on agar medium and on Bioscreen, where the viability and growth of bacteria is monitored as time dependence were used. We isolated fraction with activity against fungi *Cladosporium herbarum* and *Alternaria* sp. from *Nicotiana tabacum,* fraction with activity against *Bacillus megatherium* from *Armoracia rusticana* and fractions with activity against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* from *Pulsatilla* sp. seeds.

Acknowledgement

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SOMACLONAL VARIATION IN PLANT *IN VITRO* CULTURES AS A TOOL TO GENERATE STRAWBERRY GENOTYPES RESISTANT TO *Verticillium dahliae*

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We have demonstrated that variability occurring in strawberry *in vitro* cultures can be a source of disease resistance useful in breeding. The effective method for selecting somaclonal variants with increased resistance to *Vericillium* wilt was elaborated. The experiments were done on strawberry cultivar 'Elsanta', which is highly susceptible to the disease. The increase of frequency of somaclonal variation was achieved by adventitious shoot regeneration from dedifferentiated callus tissue. The regenerated shoots were subjected to multiple selection with homogenate of 21-days-old liquid cultures of *Verticillium dahliae.* The selected somaclones showed much higher resistance to infection with *Verticillium dahliae* in *in vitro* conditions than the original cultivar and the cultivar 'Senga Sengana', which is highly resistant/tolerant to *Vericillium* wilt in the field.

One clone, named K 40, was selected randomly from obtained resistant variants for further testing. Its resistance to *Verticillium dahliae* was re-tested *in vitro* after two passages of micropropagation and was proven to be high and stable in these conditions. Enhanced resistance of the somaclone to verticillium wilt was also confirmed on 1st, 2nd and 4th generation of plants propagated traditionally from stolons in greenhouse tests during 3 vegetative cycles. As compared to standard cultivar, plants of K 40 clone produced more biomass, their leaf petioles were longer and leaf laminas bigger whereas fruit morphology, taste and colour was not affected. This shows that enhanced resistance to *Verticillium dahliae* was co-selected with enhanced growth vigour, but the selection did not affect morphological and qualitative traits of the fruit. RAPD analysis revealed the DNA polymorphism between original cultivar and selected somaclone K 40, which suggests that this somaclonal variation has a genetic background.

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CHITINASES ARE COMPONENTS OF GENERAL PLANT DEFENSE RESPONSE AGAINST HEAVY METALS BUT CAN ALSO SHOW METAL-SPECIFIC ACCUMULATION

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Plant chitinases belong to so-called pathogenesis related proteins and have mostly been detected in plants exposed to phytopathogenic viruses, bacteria or fungi. A few studies revealed that they are also likely to be involved in plant defense against heavy metals. This work was done to monitor the accumulation of chitinases in a set of heavy-metal stressed plants. Roots of different plant species including *Vicia faba* cvs. Aštar and Piešťanský, *Pisum sativum*, *Hordeum vulgare*, *Zea mays* and *Glycine max* were exposed to different concentrations of cadmium, lead and arsenic. In each case the toxicity effects were reflected in certain levels of root growth retardation. Extracts from most stressed (500 mg.l^{-1} Pb^{2+}; 300 mg.l^{-1} Cd^{2+} and 100 mg.l^{-1} As^{3+}) roots were further assayed for chitinase activity using separation on polyacrylamide gels. Our results showed that in each combination of genotype (except for the more tolerant *Vicia faba* cv. Aštar) and metal ion there were several different chitinase isoforms significantly responsive to toxic environment when compared with water-treated controls. This suggests that chitinases are stable components of plant defense against higher concentrations of heavy metals. In addition, some isoforms accumulated in response to one but not to other metal ion, which implies that these enzymes might also be involved in a more (metal) specific mechanism in affected plants.

Acknowledgement

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IDENTIFICATION OF NEW POWDERY MILDEW RESISTANCE GENES IN *Hordeum vulgare*

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Blumeria graminis DC. f. sp. *hordei* Ém. Marchal is the obligate biotrophic fungus which causes powdery mildew in barley (*Hordeum vulgare* L.). Powdery mildew is one of the common foliar diseases in temperate climates. The control of powdery mildew disease can be achieved through the use of resistant varieties of barley. Considering the limited number or complete lack of resistance genes in cultivated barley, other *Hordeum* species have been screened for effective resistance genes to powdery mildew. A very promising new source of resistance genes to important barley diseases including powdery mildew is the progenitor of cultivated barley *H. vulgare* ssp. *spontaneum*.

Mapping of powdery mildew resistance genes by means of genetic markers utilizes the identification of markers linked to the resistance genes and the location of known genes on the barley genetic map. DNA markers are the primary tools useful for genetic mapping. The objective of our work was to map resistance genes against powdery mildew in two F_2 populations derived from crosses between the winter barley variety 'Tiffany' and the wild barley accessions PI466461 and PI466197 using simple sequence repeat (SSR) markers. Sequence tagged-site markers from target chromosomal regions were used for the development of cleaved amplified polymorphic sequence (CAPS) markers linked to the resistance genes of interest. Linkage detection was carried out with 149 plants of each F_2 population and with 120 SSRs and 18 CAPSs. Map Manager QTXb17 package software was used to construct linkage groups and establish orders and map distances for each group of markers.

A two-locus model of resistance was shown by genetic mapping of both F_2 populations. In PI466461, one gene coincided with the *Mla* locus with an expected position 8 cM proximal to the *RGH1aI1a* marker designed for the known *RGH1a* gene sequences. The other and new resistance locus derived from the wild accession was found on the short arm of chromosome 7H. It was mapped close to the sub-telomeric region of this chromosome and is flanked by the markers *Bmag0021* and *EBmag0794* at the distances of 4 and 9 cM, respectively. Until now, neither a dominant/semi-dominant major gene nor a quantitative trait locus conferring powdery mildew resistance has been located on the upper arm of chromosome 7H of barley.

In PI466197, molecular analysis revealed a highly significant linkage with the markers *Bmac0213* and *MGB402* on the short arm of chromosome 1H, which is the position consistent with the *Mla* locus. The other gene was located between the markers *Bmac0134* and *MWG878* on the short arm of chromosome 2H, which could be a newly identified locus of powdery mildew resistance. The prospect of our work is to find markers tightly linked to resistance genes so that breeders could use them for marker-assisted selection.

Acknowledgement

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PLENARY SESSION V: APPLIED PLANT BIOTECHNOLOGY

PRODUCTION OF PHARMACEUTICAL PROTEINS IN PLANTS

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The potential of 'molecular farming', production of recombinant pharmaceutical proteins using plants or animals as bioreactors, is being illustrated by a number of recombinant protein products that currently undergo clinical trials. All these product candidates have been obtained using expression processes developed during late 1970ies - early 1980ies, and all currently available 'first generation' expression methods suffer from various limitations, such as the long time frame necessary for stable transformation, the low yield obtained with stable or transient systems, biosafety concerns around open field cultivation of transgenic crops expressing therapeutic proteins, and the inability of transient systems to be scaled up. These available production platforms fall into several categories: nuclear transformation, plastid transformation, transient expression based on *Agrobacterium*-mediated delivery, and transient expression mediated by plant viral vectors.

The new generation process which we term 'magnifection', is a simple and indefinitely scalable protocol for heterologous protein expression in plants, which is devoid of stable genetic transformation of a plant, but instead relies on transient amplification of viral vectors delivered to multiple areas of a plant body (systemic delivery) by *Agrobacterium*. This eclectic technology combines advantages of three biological systems: vector efficiency and systemic delivery capabilities of an *Agrobacterium*, speed and expression level/yield of a virus, and posttranslational capabilities and low cost of a plant. Thus, the magnifection platform effectively addresses most of the major shortcomings of earlier plant-based technologies.

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PLANT REGENERATION AND PRODUCTION OF EMBELIN FROM ORGANOGENIC AND EMBRYOGENIC CALLUS CULTURES OF *Embelia ribes* BURM. F. – A VULNERABLE MEDICINAL PLANT

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Embelia ribes, an important vulnerable medicinal liana were regenerated through organogenesis and embryogenesis by using leaf explants. Leaf explants produce organogenic calli on MS medium supplemented with 1.0 $mg.l^{-1}$ 2,4-D and 0.5 mgl^{-1} BA and embryogenic calli on 2.0 $mg.l^{-1}$ TDZ and 0.5 $mg.l^{-1}$ 2,4-D on the same basal medium. Somatic embryos were induced in the same medium after 6 wk of culture. The shoot regeneration was obtained from organogenic calli on MS medium containing 0.5 $mg.l^{-1}$ TDZ and 0.1 $mg.l^{-1}$ IAA. Conversion of somatic embryos into plantlets occurred on MS medium supplemented with 0.05 $mg.l^{-1}$ TDZ. Embryogenic calli were again produced in this medium at the basal part of the plantlets. The regenerated shoots were elongated on MS growth regulator free medium. Elongated shoots were rooted on MS medium supplemented with 1.0 mgl^{-1} IBA. HPLC-UV assay demonstrated the highest embelin content (53.3 mg. g^{-1} d.w) in the embryogenic callus cultures of this plant. Embelin content in organogenic calli (45.2 $mg.g^{-1}$ d.w) and the dry berries (43.3 $mg.g^{-1}$ d.w), were the embelin found naturally in this plant were also demonstrated.

CHANGES IN EXPRESSION OF *dbat* AND *dbtnbt* GENES INVOLVED IN PACLITAXEL BIOSYNTHESIS DURING GROWTH CYCLE OF *Taxus baccata* L. CALLUS CULTURES

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Paclitaxel (Taxol®, Bristol-Myers Squibb Co.), a complex diterpenoid with a unique tumour-supressing mechanism (Cragg *et al*., 1993). Recently, a large-scale culture process was developed for a paclitaxel production (Tabata, 2006). However, the commercial feasibility of a large-scale bioprocess of *Taxus* cell cultures in the near future will probably depend on detailed understanding of the taxane biosynthetic pathway, the possibilities to influence the production rates of desirable taxanes by overexpression of genes controlling slow steps, by suppressing the production of undesired taxanes or by redirection of the pathway for the production of novel taxanes with greater range and potency of anticancer activity and decreased side-effects (Jennewein and Croteau, 2001).

The aim of this work was focused on the study of expression of two genes coding for the key enzymes of the latter steps of paclitaxel biosynthetic pathway throughout the growth cycle of *Taxus baccata* L. callus cultures, dbat and dbtnbt.

The time-course of the transcriptional activity of dbat and dbtnbt and taxane accumulation in the cells within a subculture were investigated. HPLC proved traces of baccatin III and low intracellular content of paclitaxel (up to 9 $\mu g.g^{-1}$ DW). The gene expression was quantified using a quantitative real time RT-PCR in 64-day interval of subculture. The level of expression of both genes gradually increased until the stationary phase of growth cycle, with an exception of the first day. While the highest amount of dbat gene transcript was detected on the day 1 after inoculation followed by rapid decrease, the initial level of dbtnbt gene expression was not so high. Although the increase in transcriptional activity of dbat and dbtnbt positively correlated with callus growth, the intracellular accumulation of paclitaxel decreased steadily until late linear or stationary phase. The increase of the steady-state mRNA level of dbtnbt gene was followed by the paclitaxel accumulation with a delay of approximately 14 days. The paclitaxel content in callus cells did not completely correlate with the level of dbtnbt gene expression, but presumably was a result of the transcriptional activity of the genes of the downstream steps of pathway, the level of intermediates, as well as the accumulation possibilities in cells or the ability of cells to release the secondary metabolites in the culture medium.

Acknowledgement
This work was supported by grant VEGA 1/1273/04.

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THE DUCKWEEDS IS A PERSPECTIVE PRODUCTION SYSTEM FOR MOLECULAR FARMING

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Lemna minor and *Wolffia arrhyza* belongs to duckweeds, *Lemnaceae*, mono-cotyledonous family. Duckweed fronds are free-floating photosynthetic organs with measure of diameter from 1 or less to several mm and have a preferable vegetative propagation. We developed a high-efficient protocol for genetic transformation of duckweeds based on agrobacterial gene transfer for the expression and the secretion of biologically active polypeptides. Fast clonal propagation, high growth rate (biomass doubling time is 1-3 days) small size and high protein content (from 15 to 45% of dry weight depending on cultivation conditions) make duckweed an ideal for production of recombinant proteins.

Aseptic culture of duckweed plants was obtained by surface sterilization of native fronds by 0.2% $HgCl_2$ and maintained in liquid MS (Murashige & Skoog) medium with 20 $g.l^{-1}$ sucrose, pH 5.8 (for *L. minor*) and liquid Schenk & Hilde- brandt medium with 10 $g.l^{-1}$ sucrose, pH 5.8 (for *W.arrhyza*), at 23-26 °C with 16-h photoperiod.

To develop an efficient regeneration system necessary for genetic transformation we establish embryogenic callus tissue of *L. minor*. Callus induction occurred after cytokinine (TDZ or BA) pretreatment on the medium supplemented with 2,4-D. Its frequency changed in the range of 6% to 90% depending on the procedure. Calli were subcultivated monthly and used for further experiments. Regeneration of whole *Lemna* plants was obtained on medium containing BA with frequency of about 70%.

The constructs pVec035*GUS* or pVec*Act1GUS* in *Agrobacterium* strains EHA105 and AGLO (Fig. 1) were used for genetic transformation. Embryogenic calli of *L. minor* were co-cultivated with in liquid MS medium during 4-6 days. Then the calli were placed onto solid regenerating medium containing bacteria eliminating (cefotaxime) and selective (hygromicyn) agents. *W. arrhyza* were co-cultivated with *Agrobacterium* in liquid SH medium during 20 minutes. Then the plants were placed onto solid medium containing bacteria eliminating agent (cefotaxime). Co-cultivated callus of *Lemna* and plants of *Wolffia* showed high transient expression of GUS. The regenerated *Lemna* plants were analyzed histochemically (Fig. 2, 5), tested by PCR and RT-PCR (Fig. 3, 6). Absence of agrobacterial contamination in transgenic plant tissues was confirmed by PCR. Fluorimetric probe showed that 35S promoter is more effective for *L. minor* then Act1 (actine) promoter from rice (Fig. 4). We obtained 40 independent transgenic lines *L.minor* with pVec035*GUS* construct, all of them showed stable GUS-expression. From 18 independent transgenic pVec*Act1GUS* duckweed lines only 10 clones showed GUS-expression in spite of the gene presence. 35S promoter seems to be preferable in comparison with Act1 promoter.

We observe GUS expression in *W.arrhyza* plants past 3 weeks after transformation (Fig. 5). The presence of β-glucuronidase gene in Wolffia lines was confirmed by PCR analysis (Fig. 6).

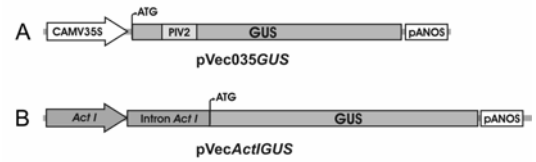

Figure 1. GUS-constructs used for transformation:
A- pVec035*GUS* encoding GUS gene under control of CMV35S promoter
B - pVec*Act1GUS*, encoding GUS gene under control of Act1 promoter.

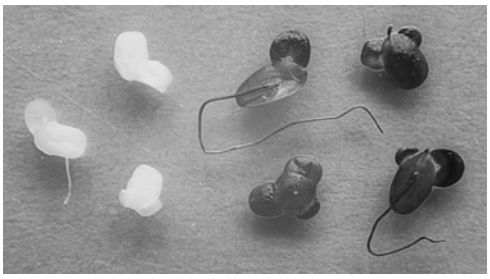

Figure 2. Control (left) and transformed (right) plants after histochemical GUS-staining (washed by ethanol).

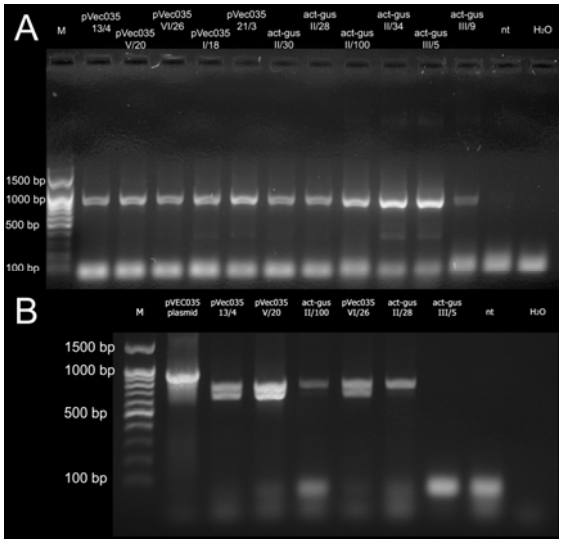

Figure 3. PCR (**A**) and RT-PCR (**B**) analyses of transgenic *Lem- na* clones, nt - control, pVec035 - lines with pVec035*GUS*; act-gus - lines with pVec*Act1GUS*

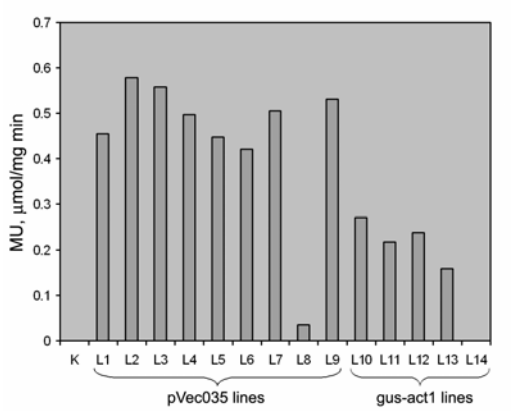

Figure 4. Fluorometric analyses of GUS activity in transgenic duck-weed lines: K-control plant, L1-L14 – transgenic lines

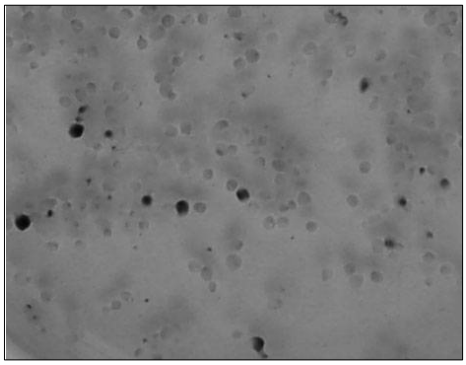

Figure 5. *Wolffia arrhyza* - 3 weeks after transformation. Histochemical GUS -staining (washed by ethanol)

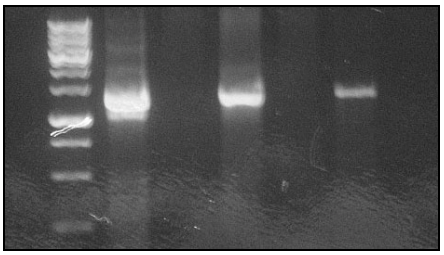

Figure 6. PCR analyses of transgenic *Wolffia* clones
nt - control, pVec035 - lines with pVec035*GUS*

ATOM FOR PEACE AND PROSPERITY IN FOOD AND AGRICULTURE

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The award of NOBEL PEACE PRIZE, 2005 to the International Atomic Energy Agency, Vienna is also the recognition of peaceful applications of nuclear techniques in food and agriculture. Nuclear applications have contributed greatly to sustainable food production worldwide. The release of more than 2,300 officially released mutant varieties of different crops in more than 60 countries is clearly an indicator of their pivotal role in enhancing the food production and socio-economic status of the growers. *In vitro* culture techniques together with nuclear technology is effective in generating genetic variability, selection of useful mutants and their multiplication in large numbers, especially in vegetative propagated crops. Radiation treatment of somatic embryogenic cell suspension is suitable for mutant selection and plant regeneration. For example, bayoud disease resistant date palm plants have been regenerated, which are already under field trials. In banana, black sigatoka disease tolerant mutant lines are under field trials for the final confirmation of the selected trait before releasing to the farmers. In cereals and other crops, anther culture is done for haploid production for doubled haploid breeding and mutagenesis. By this approach, salt tolerant wheat and rice mutant varieties have been released. Salt tolerant varieties are already released to the farmers and performing very well in China. Rice mutant varieties are being tested on multi-location. Nuclear techniques could also be effective in developing mutant lines efficient in bio-energy production, *e.g.* bio-ethanol and bio-diesel. A mutant sweet sorghum variety has already been developed in China that has 20% more sugar, and is quite useful as food, feed and bio-energy crop. Mutagenesis has a great potential in producing value added biomass for bio-energy in years to come.

NUTRITIONAL VALUE OF CEREALS; A FUNCTIONAL GENOMICS APPROACH TO UNDERSTAND THE CONTROL AND REGULATION OF AMINO ACID BIOSYNTHESIS IN BARLEY GRAIN

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The aim of the study was to obtain an insight into amino acid and storage protein metabolism in the developing barley grain at the molecular level and thereby provide a foundation for plant breeding towards improved nutritional quality. Our strategy was to generate a developing barley grains specific microarrays where a comprehensive set of genes involved in nitrogen mobilisation, transport and amino acid metabolism were assembled. The objective was to obtain a global but focused gene expression profile related to key quality traits of barley through pathway specific analysis.

Spring barley (*Hordeum vulgare* L. cv. Barke) was grown in field plots of 19.8 m² (12 m. x 1.65 m) during the summer of 2005, in Flakkebjerg, Denmark. Individual spikes were tagged and harvested in the morning (8 to 9 p.m) at 10, 15, 18 and 25 days after pollination (DAP). To identify co-regulated genes, a distance matrix was constructed (http://www.r-project.org/) and we identified three clusters corresponding to the early, middle and late seed development (Figure 1).

We investigated the gene expression pattern inside the clusters adopting pathway specific analysis. From this a number of interesting, and novel results emerged. For example the results support the emerging alternative role for rubisco during the grain development. We also observed, for the first time, the differential expression pattern of two glutamine synthase homologues during early and mid stages of grain development. Similarly the gene associated with the proline biosynthetic pathway exhibited an intriguing expression pattern.

The study described here could provide a strong complement to existing knowledge and assist our understanding of the regulatory process that occur during grain development. Ultimately enabling the improvement of quality traits in cereals which has great agricultural importance.

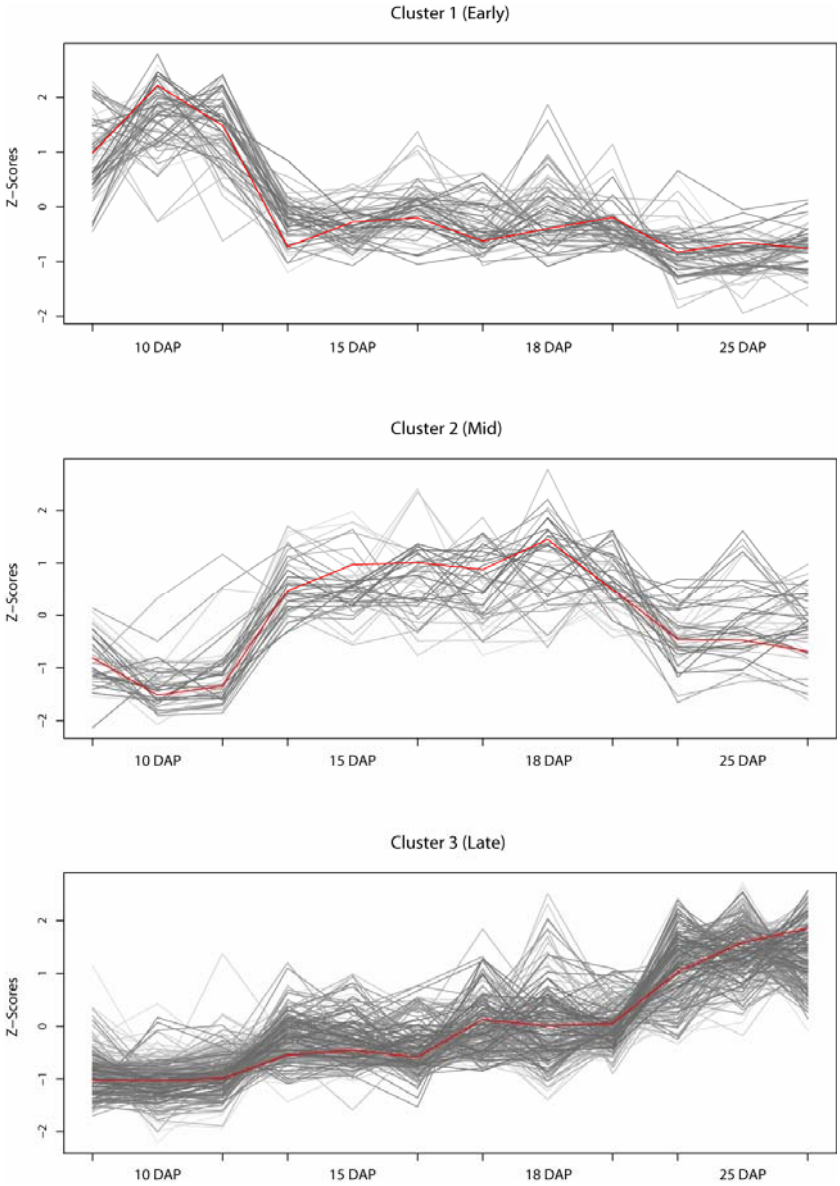

Figure 1. Cluster analysis. The gene expression profile of the 146 most significantly regulated genes representing an early-, mid- and late phase of the field-grown barley grain. The relative expression is depicted by the Z-score (transformed standard deviation) separated by the sampling time points. The red line indicated the average gene expression during development.

CEREAL FUNCTIONAL GENOMICS; THE USE OF SINGLE CELL SUSPEN- SION TO STUDY THE INPACT OF SALT STRESS

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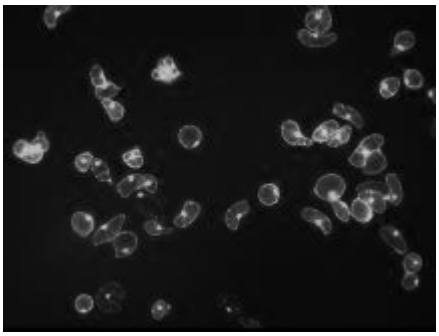
Plant sciences are rapidly moving from the genomic to the post genomic era where analysis of gene function and systems biology are becoming important ways of harnessing the huge wealth of genomic information derived for model plant species and increasingly from important crop plants for example rice. To facilitate functional genomics of monocots, in particular wheat and barley, we have developed single cell plant cultures as a model host system. The aim of the project is to study the response of genes from barley and wheat, associated with amino acid biosynthesis, when subject to different abiotic stress conditions.

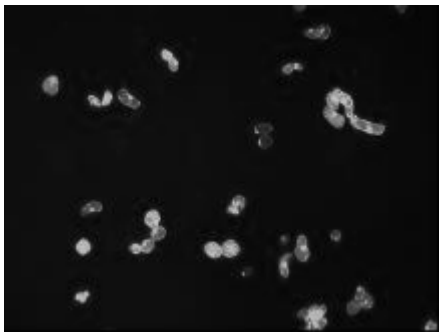
The development of the cereal suspension cultures was the first step to achieve our goal. We maintain one wheat (PC 998) and two barleys (Golden Promise and PC 1063) callus lines. The PC lines originated from the DSMZ collection, Golden Promise callus line was developed in our laboratory: 12 to 15 days old immature barley embryos were excised aseptically and undifferentiated callus formation were induced. The cell lines were maintained as callus cultures at 24 ^{o}C, in darkness. The suspension cultures were initiated from the callus cultures with approximately 50 ml of liquid medium in 250 ml Erlenmeyer flask. The microcalli were transferred into fresh liquid medium after 12-14 days of growth. The supernatant of the culture contained mostly single cells after 3-5 transfers (Figure 1).

We established growth conditions to allow routine culturing of somatic plant cells in 24 well microtiter plate format. The conditions and procedures were optimised to allow the cell cultures to be subjected to environmental stress, namely osmotic stress by NaCl. As proline is an important osmoprotectant of the cereal cells, colorimetric assay for proline detection were developed for small volumes (200µl). We performed RNA preparation followed by cDNA synthesis and RT-PCR experiments to study further the proline metabolic pathways by detecting the change of expression of the *P5CS* and *PC5R* genes as these genes and their products are involved in the biosynthetic pathway of proline. The results will be discussed.

Wheat (PC 998)



Barley (PC 1063)



Barley (Golden Promise)

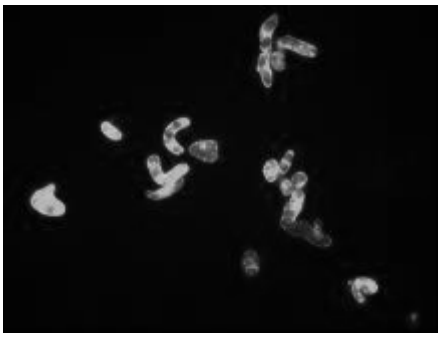


Figure 1. Cereals single cell cultures: the viability was visualised by fluorescein diacetate (FDA) staining

SEED OF LEGUMINOUS CROPS AS A PLATFORM FOR EXPRESSION OF PHARMACEUTICAL PROTEINS

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Vaccine antigens can be produced cost-effectively in feed plants. If sufficient expression level is achieved, the material can be delivered without extensive purification, further reducing the costs. Moreover plant derived antigens are inherently safer than native or other recombinant alternatives. In the past decade there has been a substantial progress in obtaining higher expression and accumulation levels of foreign proteins in the plant tissue. There are significant advantages in directing the expression of the recombinant vaccine protein into the plant seed. Plant seeds are low in water and thus offer high stability and natural microencapsulation. Seed of leguminous crops is naturally programmed to store large amounts of proteins. In soybean proteins account for up to 40% of seed dry mass. Here we report stable expression and accumulation of two vaccine antigens (B-subunit of *E.coli* heat labile enterotoxin and Human papillomavirus cpasid protein) in transgenic soybean seed. With the system described expression levels of approximately 10 g of antigen per 1 kg seed have been achieved with the potential for improvement.

Acknowledgement

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PLANT BIFUNCTIONAL NUCLEASES AND ANTINUCLEASES: CLONING, CHARACTERIZATION, EXPRESSION AND PROSPECTS OF BIOTECHNOLOGICAL AND MEDICINAL UTILIZATION

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Plant nuclease I (bifunctional nuclease) (E.C.3.1.30.x) is a sugar non-specific endonuclease, which belongs to enzymes capable of degrading double and single stranded nucleic acids. These enzymes have been reported to comprise a major nuclease activity in a number of plant species. It plays various functions including the participation in an apoptosis. The main goal of our study is to isolate and clone new homologues of plant nuclease I, analyze their expression in transformed and silenced plant lines and estimate their potential as antitumorogenic agents. Previously we published some biological effects of mung bean nuclease I on animal systems (Souček *et al*., 2006).

Recently we cloned plant nuclease I homologue involved in pathogenesis induced by plant viroids in tomato. The tomato bifunctional nuclease (TBN1) gene was obtained using RTPCR and a high fidelity PCR-based methods with conserved primers in combination with 5´ and 3‘ RACE reactions (Matoušek *et al*., 2007). In the present work, this gene was cloned into plant vector pLV-07 constructed earlier (Matoušek *et al*., 2006) and used for an *A.tumefaciens* infiltration into *Nicotiana benthamiana* young leaves to overproduce this nuclease. Subsequently, the method of TBN1 purification was developed. TBN1 was extracted from infiltrated leaf sectors with Tris buffer pH 7.5. Subsequently, proteins of the extract were fractionated with ammonium sulate from 35 to 85 % of saturation. These fractions were collected for further purification using two steps of chromatography - ion exchange and affinity chromatography. This process resulted in a fast purification of TBN1 in a homogenous sample containing probably two glycosylated forms of this enzyme, small and large one, having apparent molecular masses of about 36 and 34 kDa, respectively. Properties of purified recombinant nuclease were studied. It was found in our experiments that TBN1 has pH optima 6.3, 6.3 and 5.9 for digesting of ssRNA, ssDNA and dsDNA, respectively. Surprisingly, this nuclease does not exhibit any preference for single-stranded substrates, unlike to majority of nuclease I homologues, suggesting that TBN1 is in fact a novel homologue of plant bifunctional nuclease.

According to our results, plant infiltration/two step chromatography purification system is a procedure that enables production of nuclease for further biomedicine experiments. About 10 mg of purified nuclease we isolated from 100 g of *N.benthamiana* leaves. Our first results show that TBN1 is active in induction of aspermatogenesis and inhibition of human melanoma tumour proliferation on mice. These experiments are in progress. Besides analysis of biological effects on animal systems, a blocking TBN1 using antinucleases based on plant VIGS and antisense vectors is studied. Preliminary results show that antinucleases block in some extent plant apoptosis and aging. This finding could be used in plant biotechnology.

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DEGRADATION OF NITROESTERS BY WETLAND PLANTS

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Organic molecules containing nitrate groups are manufactured primarily for explosives or for their pharmacological effects. Because the production of most widespread explosive TNT was stopped in Czech Republic last century we concentrated on wastewater contaminated by nitroesters. Nitroglycerin (TNG, NG) is used in manufacture of dynamite, gunpowder, and rocket propellants, and as a therapeutic agent primarily to alleviate angina pectoris. Ethylene Glycol Dinitrate is an explosive ingredient (60-80%) in dynamite along with nitroglycerine (40-20%). One of inexpensive approach for cleanup of soil and water contaminated with these propellants is the use phytoremediation. Phytoremediation is the use of vegetation for *in-situ* treatment of hazardous wastes. To achieve the goal systematic approach concerning the selection of plants and optimalization of remediation processes is urgently required.

Little research has been conducted on the phytotoxicity of nitrate esters. This may be due to their lower toxicity to humans and relatively high biodegradability (compared with other explosives such as TNT). Goel *et al*. (1997) demonstrated the possibility of incubation of cell culture of *Beta vulgaris* with 454 $mg.dm^{-3}$ of TNG. We tested the phytotoxicity of nitroesters using the test based on the comparison of length of mustard primary roots after germination of seeds for 3 days on the medium supplemented with tested compounds in different concentrations. The concentration of 400 $mg.dm^{-3}$ TNG absolutely suppressed the germination of all seeds (Figure1).

In our laboratory study, the uptake and transformation of NG and EGDN from water by wetland plants was investigated using in vitro regenerants of *Juncus inflexus* and *Phragmites australis*. Plants were able to clean up water containing 500-600 $mg.dm^{-3}$ of NG or EGDN. Degradation of nitroesters by microorganisms systems involves sequential denitration steps resulting in multiple partially denitrated products (White et al., 1996, Ye et al., 2004, Snape *et al*., 1997). We supposed similar mechanism in plants. Degradation products (DNG and MNG) were identified in the medium as we expected (Figure 2).

Acknowledgement
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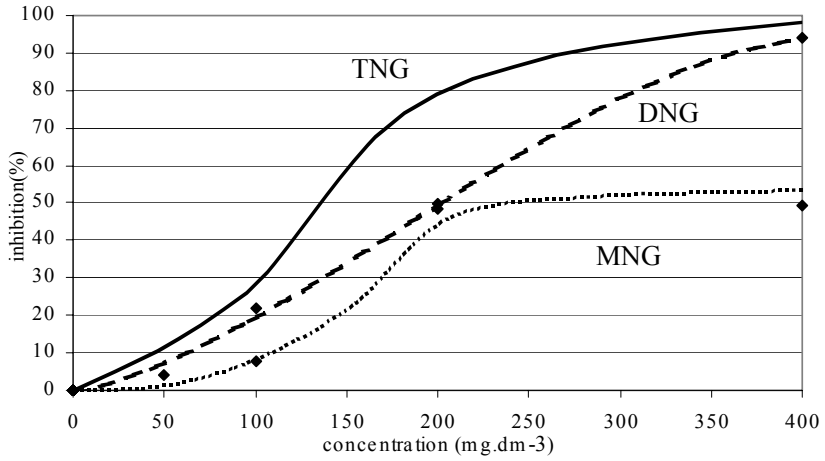

Figure 1. Inhibition of growth of roots of mustard seeds caused by treatment of nitroesters

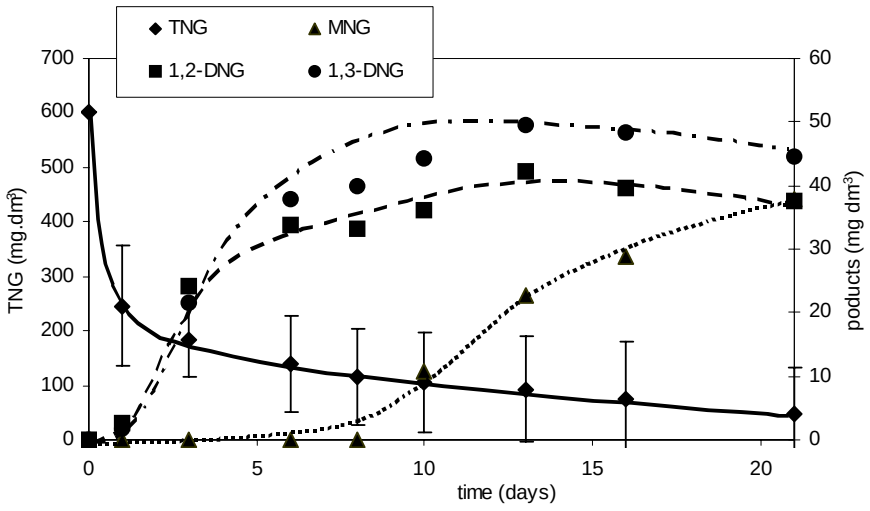

Figure 2. Degradation of TNG by in vitro regenerants of *Juncus inflexus*

A PROTEOMICS PERSPECTIVE ON CARBON ASSIMILATION IN OIL-SEEDS

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In oilseeds, protein and oil accumulate during a cell expansion phase of seed development commonly referred to as seed filling. To characterize seed filling from the perspective of protein expression, seed proteins of soybean (*Glycine max* L., var. Maverick) and oilseed rape (*Brassica napus* L.) were quantitatively analyzed during seed-filling phase of seed development using two-dimensional gel electrophoresis. Tryptic peptides from each protein spot were analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry and/or by liquid chromatography tandem mass spectrometry (Hajduch *et al*., 2005, 2006). The preponderance of these protein classes enabled the development of a high-resolution proteome model for carbon assimilation. Among many conclusions, this model reveals that sugar mobilization through glycolysis, although collaboration between the cytosol and plastids, is principally performed in the cytosol.

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POSTERS

IN VITRO CULTIVATED *Dionaea muscipula* AS A MODEL FOR INVESTIGATION OF CADMIUM DETOXIFICATION BY MEANS OF PHYTOCHELATINS

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Venus flytrap - *Dionaea muscipula* Ell., is a carnivorous plant widespread in fens and swamps of two states of USA - South and North Carolina. These plants are routinely cultivated in *in vitro* conditions for many years. They are characteristic by high regenerative capacity; so it can be used well defined *in vitro* conditions to obtain a big amount of homogenous plant material. *In vitro* cultivated plants of *Dionaea muscipula* produce wide range of secondary metabolites, such as flavonoid compounds, tannins and naphthoquinones. Production of different types of secondary metabolites varies depending on cultivation media composition, such as macro- and microelements. Sulphur plays many roles in physiological processes of plants. It is a precursor of sulphuric amino acids, such as cysteine and methionine, and from these amino acids are composed thiol compounds - glutathione and phytochelatins that play an important role in detoxification processes of heavy metals. As sulphur sources in cultivation media sulphates in the concentration 1-3 mM, especially in the form of magnesium sulphate, can serve.

This explantate culture was used to study the cadmium detoxification by phytochelatins and for study of influence of such stress conditions on synthesis of naphthoquinones. *Dionaea muscipula* explantates were cultivated for 12 weeks on unmodified MS cultivation medium as well as on modified, where all macro- and microelements in the form of sulphates were replaced by appropriate chlorides in the same concentrations. These cultivation media were enriched by cadmium in the form of cadmium chloride and cadmium acetate in the concentrations 0, 1, 5, 10, 25, 50, 100, 250, 500 and 1000 μM. The influence of cadmium acetate or cadmium sulphate on physiognomy and growth characteristics of *Dionaea muscipula* explantates was attentively investigated. Important changes in leaf anatomy and morphology were determined as well as growth suppression. The content of free and bound cadmium, thiol compounds and very important secondary metabolite of naphthoquinone group - plumbagine, was determined by using different analytical methods. Biosynthetic suppression of thiol compounds was determined according to cadmium concentration and sulphur content in cultivation media. All results acquired in this study provide the clarification of sulphur and sulphur compounds influence on heavy metals detoxification, protective compounds and secondary metabolites biosynthesis.

Acknowledgement

This work was supported by grants: 1M06030, IGA MZ 1A/8666-3 and GACR 522/07/0692.

CHANGES IN LEVELS OF THIOLS AT EMBRYONIC CULTURES OF SPRUCES EXPOSED BY CADMIUM(II) IONS

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Plants are continuously exposed to abiotic and biotic stresses in their environment. Their growth and development are closely associated with the ability of plants to respond and adapt to external stresses. Plants respond to pathogen attack and/or external stresses by marked changes in gene expression, resulting in the de novo syntheses of specific peptides and proteins such as glutathione and/or phytochelatins (Cobbett, 2000). Glutathione (GSH) plays an important role in detoxifying of toxic heavy metals and scavenging of reactive oxygen species. Moreover, the structure of GSH (a tripeptide with the sequence γ-Glu-Cys-Gly) is strongly related to that of phytochelatins (PCs), because PCs could be synthesised from GSH. PCs, small peptides consists of 4-23 amino acids, participate in the detoxification of heavy metals. The very wide scale of various analytical techniques including both chromatographic coupled with different detectors and stationary ones such as electrochemical methods has been utilizing for determination of thiols in samples of interest (Kizek *et al*., 2004; Petrlova *et al*., 2006; Vacek *et al*., 2004).

The main aim of our work was to utilize adsorptive transfer stripping technique coupled with differential pulse voltammetry Brdicka reaction to determine thiols in early somatic embryos of Spruces (ESEs) treated with 0, 250 and 500 μM concentrations of cadmium(II) ions as EDTA chelate for twelve days. If we analysed standard of GSH by Brdicka reaction, we obtained four signals called as RS2Co, Cat1, Cat2 and Cat3. For the qunatification purposes we used Cat3 signal. The linear part of this dependence was observed in the concentration range of 0 to 10 $\mu mol.l^{-1}$ of GSH ($y = 0.6419x + 0.1628$; $R2 = 0.9961$). After that, we analysed the ESEs sample by AdTS DPV Brdicka reaction. Potential of RS2Co signal of ESEs treated by Cd-EDTA was shifted to more positive potential (about 200 mV) in comparison with potential of RS2Co signal of non-treated ESEs. It clearly follows from the results obtained that content of GSH was proportional to dose of Cd-EDTA and time of treatment. GSH content increased up to 148 ± 6 $ng.mg^{-1}$ (clone 2/32) and 158 ± 7 $ng.mg^{-1}$ (clone PE 14) after twelve days long treatment.

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UTILIZATION OF COMPUTER IMAGING AND NUCLEAR MAGNETIC RESONANCE TO INVESTIGATE EFFECTS OF HEAVY METAL IONS ON EMBRYONIC CULTURES

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Studying the growth and viability of biological models of interest under this stress is needed to better describe the effect of such stress factors. It seems to be that image analysis could offer more regardful tool used for studying of cell growth without any damaging and contaminating of the biological material of interest. Recently we have published paper where we used image analysis (IA) for determination of growth and viability of early somatic embryos of Spruces (ESEs) (Petrek *et al*., 2005). In the present work we utilized the image analysis for estimation of growth (increase in ESEs clusters area) of ESEs treated by lead and/or cadmium ions for twelve days. Growth of non-treated ESEs was proportional to length of the cultivation for both clone 2/32 and PE 14. The highest concentration of Pb-EDTA and/or Cd-EDTA (500 μM) caused the decrease in growth of ESEs about 20 % in comparison with control, whereas the lower dose (250 μM) caused the marked growth depression. It follows from the results obtained that ESEs grew and developed better in the presence of 500 μM of the metal ions than in the presence of 250 μM. This phenomenon probably relates with increase of ESEs clusters area by intensive uptake of water from cultivation medium due to dilution of heavy metal concentration inside the cluster. We decided to evaluate our hypothesis by means of nuclear magnetic resonance (NMR) determining content of water via protons. We found out that water content in the ESEs increased with increasing concentration of cadmium(II) ions in the cultivation medium for the 14 days long treatment. The increase was well observable during the first three days of the treatment in comparison with control. Because we estimated that the content of dry weight decreased with increasing water content and vice versa, we compared these very surprising and interesting data with the determination of dry weight. We successfully confirmed that ESEs treated with the higher concentration of Cd-EDTA had less dry weight and contained more water. In the very end of the treatment, the dry weight content in ESEs was as followed: the control ESEs contained 4.74 % of dry weight, ESEs treated with 50 μM Cd-EDTA contained 4.08 %, ESEs treated with 250 μM Cd-EDTA contained 3.34 % and ESEs treated with 500 μM Cd-EDTA contained 3.05 %. If we analysed the Pb-EDTA treated ESEs, we obtained similar results.

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CULTURES *IN VITRO* – AN ALTERNATIVE SOURCE OF PLANT SECONDARY METABOLITES

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The cultivation of medicinal plants is in the focus over the world and this attention is growing with the extension of civilization diseases, mainly cardio-vascular and cancer disorders, however, also in connection with the prevention and the nourishment of the mankind. The attention is maintained mainly to plants serving as alternative sources of bioproducts, safety natural compounds irreparable caloric eatables or natural dyes and drugs (Vanisree *et al.*, 2004). Special plant species contain considerable secondary metabolites utilized in food industry, cosmetics, and mainly in pharmaceutical industry. Besides common isolation of secondary compounds from natural sources steadily more notice is given to alternative sources of secondary metabolites, e.g. to cultures *in vitro* (Vanisree, Tsay, 2004). This possibility interesting from the medicinal, as well as environmental point of view is an effective biotechnologically utilizable source of secondary compound protecting the environment. Cell and callus cultures are a permanent source of plant biomass for secondary metabolites production. Many plant secondary products are involved in the interaction of the plant with its environment, and are inducible by biotic or abiotic elicitation (Sudha, Ravishankar, 2002).

Our study was focused on the production of pharmacologically important compounds of various structures; anthraquinones, the compounds of terpenoid character (ginkgolides, bilobalide, taxanes), rosmarinic acid, and flavonoids from the assorted plant species (*Karwinskia* sp., *Ginkgo biloba* L., *Melissa officinalis* L., *Rubia tinctorum* L., *Taxus baccata* L.) cultured *in vitro* (Table 1). We paid attention to the quantitative content of the substances presented above and to the potential possibility to increase the volume of these interesting pharmaceutical compounds at condition *in vitro*. These cultures *in vitro* produced secondary metabolites in smaller amounts than stated in intact plants (*Karwinskia* sp., *Ginkgo biloba* L., *Melissa officinalis* L., *Rubia tinctorum* L., *Taxus baccata* L.). In some cases, the concentration of tested substance in callus cultures was equivalent to its content in intact plants (*Melissa officinalis* L.). Cadmium salts, culture conditions: cultivation in 16-hour photoperiod or in the dark, growth regulators in various combinations and concentrations, and selected mono- and oligosaccharides added to culture medium have increased the production of secondary metabolites followed. The increase of rosmarinic acid production in suspension cultures of lemon balm was achieved with the addition of $Cd(NO_3)_2$ at the end of the second subculture. In this case, the content of rosmarinic acid was comparable with its content in intact plants. $CdCl_2$ (0.005 $mg.l^{-1}$) in culture medium increased the content of flavonoids in madder up to 51-61 % compared to the control. The content of flavonoids was positively influenced also in the conditions of a 16-hour photoperiod and in the presence of sole NAA (4 $mg.l^{-1}$), or in combination with BAP (1 $mg.l^{-1}$). Suspension cultures of yew (*Taxus baccata* L.) have significantly increased the production and secretion of paclitaxel into the media with galactose (3 %), mannose (3 %) or cellobiose (3 %), when initiated from a 14-day inoculum. The highest paclitaxel yield in yew cells and medium

has been recorded when cultured with 2 % sucrose together with 3 % cellobiose after 42 days of culture.

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Table 1. Cultures *in vitro* of selected plant species and their secondary metabolites

Plant source	Type of primary explantat	Secondary metabolite	Effect	Type of *in vitro* culture	Author
Karwinskia humboldtiana Karwinskia parvifolia	zygotic embryo, apical meristem, stem segment	Peroxisomicine A_1	anticarcinogenic	callus, shoot	Němcová, 1999 Argalášová-Šutovská, 2000 Kákoniová, *et al*. 2000
Ginkgo biloba L.	zygotic embryo, leaf	flavonoids ginkgolide A, B bilobalide	antiageing, stress-alleviating neuroprotective	callus	Janotková, 1999 Nehnevajová, 2001, 2002
Melissa officinalis L.	stem segment	rosmarinic acid	antioxidant, antiviral, anti-inflammatory	callus, suspension culture	Šupalová 2004
Rubia tinctorum L.	leaf	flavonoids	antioxidant	callus	Juráková, 2005
Taxus baccata L.	stem segment	taxanes, paclitaxel	anticarcinogenic	callus, suspension culture	Kuliková, 2006 Kákoniová *et al*., 2006

Media: ***Murashige-Skoog*** *(1962) - Ginkgo biloba L., Rubia tinctorum L.*
Philips-Collins *(1979) - Melissa officinalis L.*
WPM medium *(Lloyd, McCown 1980) - Karwinskia humboldtiana, Karwinskia parvifolia*
Z medium *(Čierna et al. 1991) - Karwinskia humboldtiana, Karwinskia parvifolia, Rubia tinctorum L.*
Gamborg B5 *(Gamborg et al., 1968) - Taxus baccata L.*

TOBACCO BY-2 CELL CULTURE AS MODEL FOR TESTING OF ANTIOXIDANT PROPERTIES OF VARIOUS COMPOUNDS

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Plant cell suspension culture of *Nicotiana tabacum* (*Solanaceae*) cv. Bright Yellow (BY-2) represents a very important model for investigating of cytotoxic and other properties of different substances. Plant cells susceptibly respond to presence of toxic compounds in cultivation medium. Their effect is observable as changes of plant BY-2 cells viability as well as structural changes of various cell compartments, such as nuclear architecture and chromatin structure. These variances can be attached by plant cell cycle alternations in several mitosis phases. There have been published plenty of papers displaying that substances, e.g. hydrogen peroxide or camptothecin, can start processes of programmed cell death with all hallmarks, such as presence of apoptotic like bodies. Because plant cells are affected by free radicals constantly, there have been described compounds with properties to protect cells against these radicals such as flavonoids. They are a group of polyphenolic compounds widely distributed throughout the plant kingdom contributing to the antioxidant properties of green vegetables, fruits, olive and soybean oils, red wine, chocolate, and teas. Thus, the main aim of this work was to investigate the antioxidant properties of the certain flavonoids (pomiferine, isopomiferine and osajine) at BY-2 tobacco cells treated by hydrogen peroxide.

BY-2 plant cells were cultivated in the presence of the mentioned flavonoids at the concentrations of 0, 1, 10, 50, 100, 250 and 500 μM and with trolox, a compound with oxygen radical absorbance capacity with/without addition of 12 mM hydrogen peroxide. Viability changes and growth curves of untreated BY-2 cells as well as BY-2 cells treated by flavonoid compounds with/without presence of hydrogen peroxide were detected using esterase activity. The nuclear morphology, morphological changes in nuclei architecture, mitotic ratio and DNA fragmentation were observed after staining by fluorescent dye and observed by using epifluorescence microscopy. The content of thiol compounds (GSH/GSSG, phytochelatins) and enzymatic activities of glutathione-peroxidase and catalase in plant samples were also determined. All findings were correlated with antioxidant activity of tested compound using DPPH radical extinction.

Surveyed antioxidant compounds showed protective effect on BY-2 cells according to their concentration. Viability of BY-2 cells treated by combination hydrogen peroxide/flavonoids was higher as compared to BY-2 cells treated only by hydrogen peroxide. This effect relates to antioxidant properties of tested compound as shown correlation with DPPH radical extinction test. Based on the results obtained the certain flavonoids have considerable effect on BY-2 tobacco cells with respect to their antioxidation activity.

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TRANSFORMATION OF CHLOROBENZOIC ACIDS - PCB DEGRADATION PRODUCTS BY PLANT CELLS

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The use of plants for remediation of contaminated areas is possible alternative to physico-chemical methods. Plant are able to detoxify various organic compounds and at natural conditions they participate on degradation of different xenobiotics and they cooperate with other organisms present in particular ecosystem. Polychlorinated biphenyls are efficiently degraded by several bacterial species to chlorobenzoic acids. These compounds can be further metabolized to different products.

The metabolism of chlorobenzoic acids, (intermediates of bacterial polychlorinated biphenyls metabolism) in microorganisms has alredy been studied in detail. However, the knowledge of chlorobenzoic acids metabolism in plants is not complete and sufficient yet.

The aim of this work was to find out whether the cells of higher plants are able to metabolise chlorobenzoic acids and tried to find products formed in this process.

The transformation of the chlorobenzoic acids was tested using plant cells of three species cultivated *in vitro*. Embryogenic morphologically differentiated culture of *Armoracia rusticana* K54 (horseradish), amorphous callus culture of *Nicotiana tabacum* WSC 38 (tobacco) and hairy root culture of *Solanum nigrum* SNC 9O (black nightshade).

In microorganisms degradation efficiency depends on microbial species, number of chlor atoms and their position. In plants we found the similar phenomenon. The efficiency of chlorobenzoic acid transformation by tested plant species was different. Cells of *Solanum nigrum* showed high degree of 2-, 3-, 4-chlorobenzoic acids, 2,3-di, 2,4-di, 2,5-dichlorobenzoic acids and 2,3,5-trichlorobenzoic acid conversion (almost 100%) in 14 days.

Methylester of 2,3-dichlorobenzoic acid and methylester of 2,4-dichlorobenzoic acid were identified by GC-MS analysis in medium after cultivation of *Solanum nigrum* with 2,3-dichlorobenzoic acid and 2,4-dichlorobenzoic acid. In biomass after kultivation of *Solanum nigrum* with 2,5-dichlorobenzoic acid were hydroxyderivate of 2,5-dichlorobenzoic acid and 2,5-dichlorobenzoic acid identified.

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SECONDARY METABOLITE CONTENT IN SHOOTS OF *Gentianella austriaca* AND *Gentianella bulgarica* CULTURED *IN VITRO*

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Genus *Gentianella* Moench (*Gentianaceae*) comprises about 250 species worldwide. *G. austriaca* is a biennial herb 10-40 cm in height, usually branched from the base forming a corymbose inflorescence with purplish corolla *G. bulgarica* also biennial 5-20 cm in height, with long procumbent branches from the base and pale violet corolla. *G. austriaca* is well distributed in East and Central Europe and *G. bulgarica* in the central parts of Balcan peninsula. The main secondary metabolites of *Gentianella* species are xanthones, *C*-glucoflavones and secoiridoids. Phytochemical investigation of *G. austriaca* and *G. bulgarica* collected from populations growing in nature revealed 1,3,5,8-oxygenated xanthones and flavone-*C*-glucosides typical for the genus (Janković *et al*., 2005).

To the best of our knowledge, *in vitro* propagation of *G. austriaca* and *G. bulgarica* has not been reported previously. There is a single study on *in vitro* propagation of *G. albiflora* (Huo & Zheng, 2002). *In vitro* propagation of related species of *Gentiana*, *Swertia*, *Centaurium* and *Blackstonia* have been well documented (Skrzypczak *et al*., 1993 and references cited therein).

We established *Gentianella* shoot cultures from epicotyls of aseptically germinated seeds. Cultures were maintained on MS medium with 0.5 $mg.l^{-1}$ BA and 0.1 $mg.l^{-1}$ NAA. Cultures of both species manifested precocious *in vitro* flowering.

Samples for phytochemical analysis were air-dried and extracted with MeOH for 48 h at room temperature. The ratio between plant material and solvent was 1:20. Analyses were carried out on Agilent series 1100 with DAD detector, on reverse phase Zorbax SB-C18 analytical column 150 x 4.6 mm i.d., particle size 5 μm (HP). Mobile phase A (H_2O containing 1% 0.1 N H_3PO_4) + B (MeCN), elution by gradient according to the following scheme: 98-90% A 0-5 min, 90% A 5-10 min, 90-85% A 10-13 min, 85% A 13-15 min, 85-70% A 15-20 min, 70-40% A 20-24 min, 40-0% A 24-28 min. Flow $1ml.min^{-1}$, detection at 260 and 320 nm.

Xanthones demethylbellidifolin (DMB), demethylbellidifolin-8-*O*-glucoside (DGL) and bellidifolin-8-*O*-glucoside (BGL) were isolated previously (Janković, 2005). Quantification was performed using HPLC and the amounts of compounds were calculated using calibration curves. All experiments were repeted at least two times. The results are presented as $mg.g^{-1}$ of dry weight.

Three xanthones (DMB, DGL and BGL) from aerial parts of *G. austriaca* and *G. bulgarica* cultured *in vitro* quantified by HPLC are presented in Table 1. The amounts of xantone compounds in aerial parts of *G. austriaca* were nearly two times higher than in shoots *in vitro*. In *G. bulgarica* the differences in xanthone content between plants cultured *in vitro* and naturaly growing plants was much less pronounced. It is evident that the production of xanthones is affected by the concentration of BA in the medium.

In *G. austriaca*, the amounts of DMB and DGL increased with increase of BA while the BGL concentration decreased. Similar pattern can be observed in *G. bulgarica.*

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Table 1. Xanthone content of *G. austriaca* and *G. bulgarica*

BA ($mg.l^{-1}$)	Multiplication index		DGL (mg/g dw)		BGL (mg/g dw)		DMB (mg/g dw)	
	G. austr.	*G. bulg.*	*G. austr.*	*G. bulg.*	*G. austr.*	*G. bulg.*	*G. austr.*	*G. bulg.*
0.1	1.5 ± 0.1a	2.0 ± 0.2 a	11.81	4.35	9.93	4.64	0.72	1.89
0.2	2.7 ± 0.3 b	1.9 ± 0.9 a	12.43	1.88	10.19	1.64	0.98	0.21
0.5	3.1 ± 0.4 bc	2.2 ± 0.2 a	12.57	3.69	8.69	2.63	1.13	0.22
1.0	3.9 ± 0.4 c	1.9 ± 0.2 a	15.16	5.34	8.14	3.07	1.20	1.89
nature	-	-	25.6	3.53	23.04	5.82	0.86	1.89

Figure 1. *G. austriaca* – precocious *in vitro* flowering

Figure 2. *G. balcanica* shoot cultures

TRANSIENT EXPRESSION OF PROTEINS USING MODIFIED PLANT VIRAL VECTORS

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Plant viral vectors used for transient expression of proteins are an attractive alternative to conventional breeding and transgenic methodology. Transient expression is fast, flexible, unaffected by chromosomal positional effects and can be used in fully differentiated plant tissues (Fischer *et al*., 1999). Transient systems based on virus allow expression of foreign genes at higher levels in infected tissues than is normally the case in transformed plants (Yusibov *et al*., 1999).

We have modified potato virus X (PVX) based expression vector pGR106 by insertion of its modified coat protein bearing two human papillomavirus (HPV) epitopes. Epitope from E7 protein (44-60 aa) was fused either to N- or C- terminus of PVX CP and epitope from L2 protein (21-32aa) only to N- terminus. The coding sequences of these fusion proteins were obtained by PCR and cloned into the bacterial expression vector pMPM4. The constructs will be cloned also into the PVX based expression vector pGR106 and the level of fusion proteins expression in both systems will be determined. The plant expression system has the potential for use as vaccines.

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STUDY OF PHYTOREMEDIATION MECHANISM OF ORGANIC NITRO-COMPOUNDS

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Phytoremediation is the use of plants to partially or substantially remediate selected contaminants in contaminated soil, sludge, sediment, ground water, surface water, and waste water. Phytoremediation takes many advantages to which belong especially the perspective of cost reduction and very regardful stance of the environment.

The aim of this work is the study of degradation mechanism of organic nitrocompounds, especially 2,4,6-trinitrotoluene (TNT) and glyceroltrinitrate (GTN), through the use of the higher plants and its practical application. The first section of the work is focused on identification of the degradation products of organic nitrocompounds through the use of the laboratory experiments. The second section is focused on *in situ* experiments, that took place in real contaminated locality and demonstrate the ability of selected plant species to degrade organic nitrocompounds in real conditions.

The laboratory experiments proved that TNT degradation through the plant proceed mainly by reductive mechanism. It was found that the main products of phytodegradation are 4-ADNT and 2-ADNT. The oxidative mechanism of phytodegradation, that has trinitrobenzene (TNB) as the degradation product, was observed only in plant extracts of *Saponaria officinalis* and *Arabidopsis thaliana*. The study of GTN phytodegradation showed the sequential formation of expected degradation products – GDN and GMN. The formation of the supposed end product glycerol was not proved.

Within the scope of *in situ* experiments it was verified that nitroesters could be removed from waste water in real conditions during couple of days.

The last part of the study focuses on the proteome analysis of *Arabidopsis thaliana* by two-dimensional gel electrophoresis. The phytoremediation proteome is given the hundreds of enzymes already identified as a participating in bacterial remediation processes. The aim of the study is to find and identify the changes in *Arabidopsis thaliana* proteome incurred by xenobitic influence.

From the obtained results it is obvious, that the higher plants are capable to participate in resistant xenobiotics metabolism and degradation. Due to this properties it is advantageous to search perspective plant species and cultivate them for the purpose of hazardous organic compounds and toxic metals removing.

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