MOLECULAR ANALYSIS OF DH LINES OF WINTER OILSEED RAPE TRANSFORMED WITH GENES CHANGING IN RESPONSE TO DROUGHT STRESS

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Introduction

Winter oilseed rape (*Brassica napus* var. *oleifera* L.) is the most commonly grown oil plant in European Union. Due to its wide application, both for food, feed and fuel, each year the area of oilseed rape cultivation increases. The drought problem in the cultivation of rape is important and causes large losses of yield, and therefore research which would lead to an increased understanding of mechanisms of plant tolerance to drought stress is desirable for breeding.

The method of *in vitro* androgenesis is a technology that provides valuable material for breeding by reducing the production cycle and selection of desired genotypes; it is also useful in basic research, because it allows to test homozygous material. Microspore derived embryos from oilseed rape DH lines, due to high capacity for regeneration and genetic homogenous constitute very good material for genetic transformation. After genetic transformation of microspore derived embryo and doubling number of chromosomes of obtained plant, homozygous transformant is received (Cegielska-Taras T. et al. 2008).

The methods which apply *Agrobacterium tumefaciens* for *Brassica* transgenesis are widely used (Cardoza, Stewart, 2004). Mostly inserted traits genes are: resistance to herbicides, viruses, fungi, insects, environmental factors such as drought, salinity, and changes in the composition of fatty acids and proteins in the seeds (Kahrizi et al. 2007, Senior, Bavage 2003, Wang i in. 2005). That metod is also used for transgenesis for research needs, for example knowing biological processes , such as functioning of myrosinase system (Troczyńska et al. 2003).

The genetic source of resistance to drought in species of *Brassica napus* L. is unknown. Therefore there is a great need of studies aimed at discovering mechanisms which increase tolerance to drought stress and genetic manipulations which widen the range of genetic variability. The use of transfer of particular foreign genes to plant genome is an alternative path to obtain oilseed rape with new quality traits.

Plant reaction for drought stress induces a lot of biochemical, physiological and morphological changes (Figure 1.). These changes, in the course of adaptability to drought stress, are mainly conditioned by the increase of abscisic acid (ABA) level. The key role in regulation of signaling the content of ABA is attributed above all to phosphatases ABI1 and ABI2 (ABA Intensive). ABI1 is a negative regulator of ABA. The higher activity of ABI1 phosphatase increase sensitivity to stress (Ludwików, Sadowski, 2008).

Concentration of calcium (Ca) in cytoplasm increases substantially in response to some factors of stress. The information about stress (an increase in the calcium concentration) is sensed by numerous proteins, including calcium dependent protein kinases, CDPK. Analysis of transgenic rice with overexpression of CDPK7 gene, confirmed higher drought tolerance. The comparison of sequences confirmed similarity of CDPK7 from rice and CDPK6 from A. thaliana (Misztal et al. 2004). We expect that overexpression of CDPK6 in oilseed rape cause higher drought tolerance.

Materials and Methods

Plant material included DH lines of winter oilseed rape selected in drought tolerance tests. Embryos for transformation were obtained from isolated microspores of winter oilseed rape DH-lines: M-155, MI-305 and cultivar MONOLIT according to Cegielska et al. (2002).

Agrobacterium tumefaciens strain LBA4404 contains a binary plasmid pKGIB (with *ABI1* or *CDPK6* gene) and was used for transformation. Vectors contained the *bar* gene determining resistance for phosphinotricine herbicides.(Figure 2.)

The selection of explants after transformation was carried out on the medium with phosphinotricine. To determine ploidy of regenerated plants the nuclear DNA content was examined using flow cytometer. The number of chromosomes of haploid plants was doubled with colchicine. After vernalization transformants were grown in the greenhouse. Genomic DNA from regenerated T0 plants was isolated using modified Doyle and Doyle method (1990). Then it was analyzed for the presence of the transgene using Polymerase Chain Reaction with primers specific to the introduced genes. PCR products were analyzed in agarose gel and compared to 100bp DNA ladder as a molecular weight pattern.

The seeds of T1 generation were sown and a few day seedlings were examined for the presence of transgene using PCR as well. Confirmation of homozygosity for each transgenic line was the detection of the transgene in all analyzed seedlings from seeds of the T1 generation.

Homozygous lines were analyzed in terms of number of copies of a gene introduced through Southern hybridization. Genomic DNA from T1 plants of transgenic homozygous lines was isolated according to the modified Doyle and Doyle method (1990). 20 µg of DNA was digested with *Bam*H I, *Eco*R I, *Eco*R V and *Hind* III enzymes and after electrophoresis blotted onto a nylon membrane (positively charged). As a molecular probe, PCR product of *bar* gene amplification was taken and prepared with DIG labeling kit. Detection of a signal was obtained by CDP-Star® reagent chemiluminescence and exposure membrane to X-ray film.

Results and Discussion

Microspore-derived embryos seem to be good material for transformation, because of high potential for regeneration (Cegielska-Taras et al. 2008). Using diploid explants like: leaf discs, cotyledons, hypocotyls, protoplasts makes a plant chimerical for the inserted transgene. Researchers routinely use hypocotyl explants from 5-8 day old seedlings to transformation, but that procedure needs two generations of plants to select homozygous plants (Troczyńska et al. 2003). Because of the integration, T-DNA with haploid cells, explants, like microspore-derived embryos, seems to be a better transformation material. After colchicine treatment transgened genome is doubled. Transformation of microspore-derived embryos increases probability to obtain homozygous transformants (Cegielska-Taras et al. 2008).

After regeneration and selection on a medium with phosphinotricin, 80 plants were obtained, 28 after transformation with *ABI1* gene and 52 with *CDPK6.* After PCR analysis 9 of them turned out to have an inserted gene. Plants with positive PCR screening test were: one tetraploid, five diploids and three haploids. Next generation seeds were obtained from all plants. Homozygosity was confirmed by PCR in 6 DH lines (a tetraploid wasn't taken for analysis).The example of the results of confirmation is presented in Figure3.

Homozygous 6 lines were analyzed in terms of number of copies of a gene introduced through Southern hybridization.

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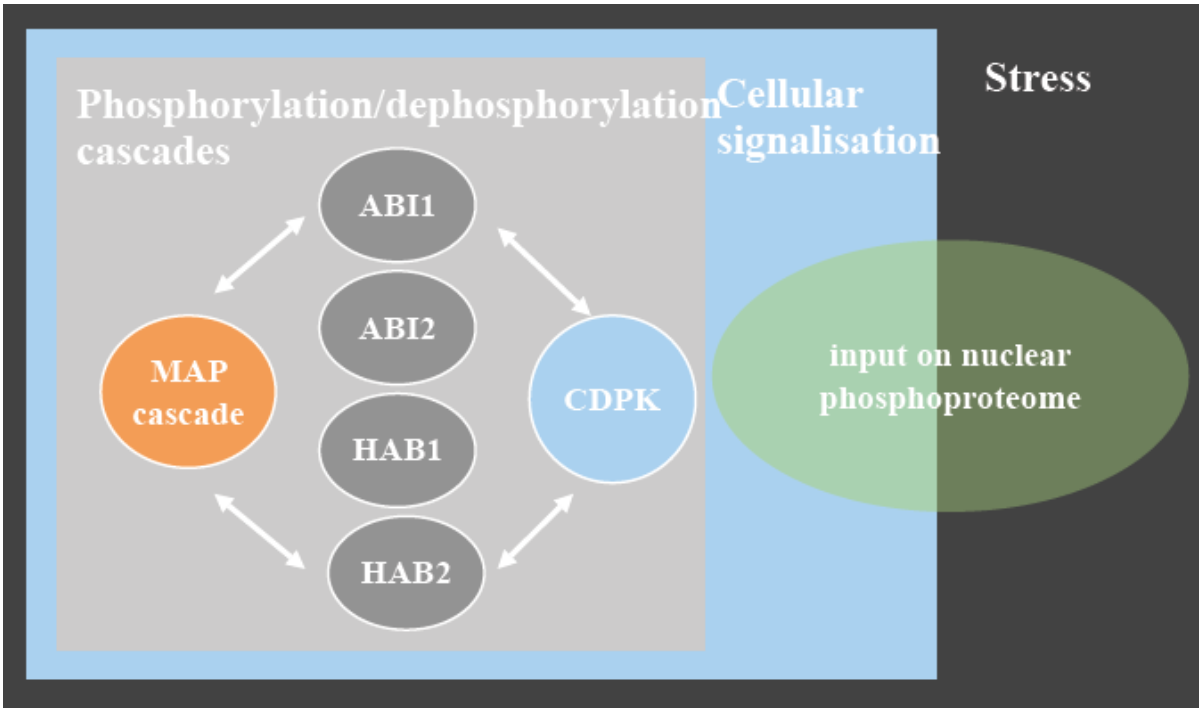

Figure 1. Signal cascades in cellular stress response. (Author: A. Ludwików)

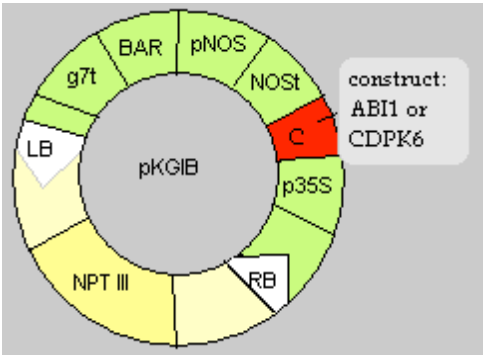

Figure 2. Plasmid pKGIB - the T-DNA organization.

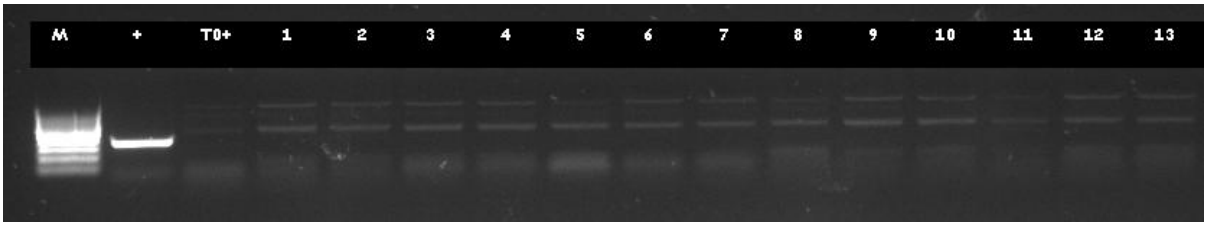

Figure 3. PCR analysis of the *bar* gene in T1 plants of DH 41 transformant of oilseed rape cv Monolit. Lanes: M-100bp DNA ladder; + - plasmid pKGIB, positive control; T0+ - 41-T0 transformant, positive control; 1-13 – T1 plants of DH 41 transformant