Editing of the MYB genes in *Brassica napus* as a method to increase anthocyanin pigmentation and stress tolerance

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Abstract. Anthocyanin hyperaccumulation is an important agricultural trait, associated with resistance to abiotic stress, pests, phytopathogenic fungi and bacterial diseases. *B. napus* with increased anthocyanin pigmentation can be generated by genome editing. Many transcription factors of the MYB family are involved in stress response and anthocyanin biosynthesis. Genes *AtMYB60*, *AtCPC* and *AtMYBL2* are negative regulators of anthocyanin biosynthesis in *Arabidopsis*, therefore the knockout of these genes can result in increased anthocyanin pigmentation. gRNA spacers were synthesized to target the orthologs of these genes, identified in *Brassica napus*. Resulting genetic constructs were introduced to the plant tissues by agroinfiltration. Transient expression of gRNAs targeting DNA-binding domains of MYB transcription factors along with Cas9 nuclease successfully promoted anthocyanin hyperaccumulation. These genetic constructs can be used for genome editing and production of new colored and stress tolerant varieties of oilseed rape.

1 Introduction

Brassica napus L. (oilseed rape, rapeseed) is one of the most important crops in the world. It is used as a source of vegetable oil and protein, animal feed and biodiesel. Rapeseed is also a good cover crop [1]. Despite cold tolerance, it is highly susceptible to fungal diseases (powdery mildew, light leaf spot, alternaria) and pests (cabbage-stem flea beetle, cabbage leaf and flower beetle) [2]. Great amount of pesticides are used to protect the yields. Canola, which is the rapeseed with low levels of erucic acid, belongs to the 'big four' biotech crops. Genetically modified canola, resistant to herbicides and insects, have been adopted in recent years [3]. High oleic acid canola is the only alternative variety. Wide use of pesticides brings harm to bees and other important organisms and promotes the development of pesticide-tolerance in weeds and pests. There have never been an attempt to enhance natural stress response mechanisms of *B. napus* on molecular level, however it is a more effective and environmentally friendly method to increase the yields.

Genome editing (CRISPR/Cas9, in particular) is a novel technique which allows to make precise modifications of the DNA [4]. The knockout and overexpression are

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frequently used methods to study gene functions in *Arabidopsis thaliana*, which is a famous model plant and a close relative of *B. napus*. But the role of many genes of rapeseed is not yet verified because of a large tetraploid genome and gene duplications. Genetic transformation of *B. napus* is also a problematic task. Each variety requires special regeneration medium and hormones. *In planta* transformation [5] and agroinfiltration are possible for this species, but these methods are rarely used and require optimization. Gene knockout in *B. napus* can be accomplished with universal vectors [6] which only require the insertion of a guide RNA spacer, complementary to the gene of interest.

Many genes are involved in the anthocyanin biosynthesis pathway in plants. Structural genes are regulated by three types of transcription factors: with the MYB domain, with the helix-loop-helix domain (bHLH) and with WD repeats [7]. Some of these transcription factors act as positive regulators of anthocyanin biosynthesis, and some are negative regulators. Hyperaccumulation of anthocyanins is associated with resistance to abiotic stress, pests, phytopathogenic fungi and bacterial diseases [8-11]. The knockout of the negative regulators of anthocyanin biosynthesis could increase not only pigmentation, but also stress-tolerance of the plants. Genes of MYB transcription factors, namely *AtMYB60* [12], *CPC* (At2g46401) [13] and *AtMYBL2* [14], were reported to suppress anthocyanin accumulation in *A. thaliana*.

Silencing of the *MYBL2* gene [14] promoted anthocyanin pigmentation of *A. thaliana*. Mutants with *AtMYB60* gene knockout demonstrated increased resistance to drought, and heterologous expression of *AtMYB60* in lettuce [12] repressed anthocyanin accumulation in the leaves. Overexpression of *CPC* gene in *A. thaliana* reduced anthocyanin accumulation under different stress conditions [13]. These manipulations did not have any negative effect on the fitness of the plants.

In this study we demonstrate that transient knockout of *MYB60*, *CPC* and *MYBL2* genes in the leaves of *B. napus* can promote anthocyanin pigmentation.

2 Materials and methods

2.1 Biological material

Spring variety of rapeseed ‘Ratnik’ was used in the experiments. Seeds were sown on a universal soil (Geolia, Russia) and grown under 10000 lux illuminance provided by LED phytolamps in the short-day conditions (10-hr light/14-hr dark) at 20°C. Plants were used for the experiments at the age of 3-4 weeks.

XL-blue strain of *E.coli* was used for the cloning of plasmids.

Agl0 strain of *Agrobacterium tumefaciens* was used for the agroinfiltration. It was demonstrated that this strain can successfully infects *B. napus* variety ‘Ratnik’[5].

2.2 gRNA spacer design

Cross-pollination and interspecific hybridization are common in *Brassicaceae* plants, therefore targeting the most conserved and essential gene regions should increase the chances of the knockout. *B napus* orthologs of the target genes *AtMYB60* [12], *CPC* (At2g46410) [13] and *AtMYBL2* [14] and their conserved domains were found using OrthoDB catalog [15] and BLAST. gRNA spacers for the knockout were designed using CRISPOR software [16]. Chosen oligonucleotides containing the gRNA spacer sequences were synthesized by Evrogen (Russia) according to Čermák et al. [6].

2.3 Plasmid construction

Plasmids for the knockout of target genes *MYB60*, *CPC* and *MYBL2* were created by cloning gRNA spacer oligonucleotides into the pDIRECT_23A vector [6], which was a gift from Daniel Voytas (Addgene plasmid #91138). It consists of gRNA scaffold, gene encoding Cas9 nuclease and Basta selectable marker (fig. 1). Phosphorylated oligonucleotides were introduced to the vector instead of the *LacZ* gene using Golden Gate method. Resulting plasmids were isolated from *E. coli* with GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) and sequenced using primer GGAATAAGGGCGACACGGAAATG. Genetic maps of the plasmids were generated using UGENE software (fig. 1).

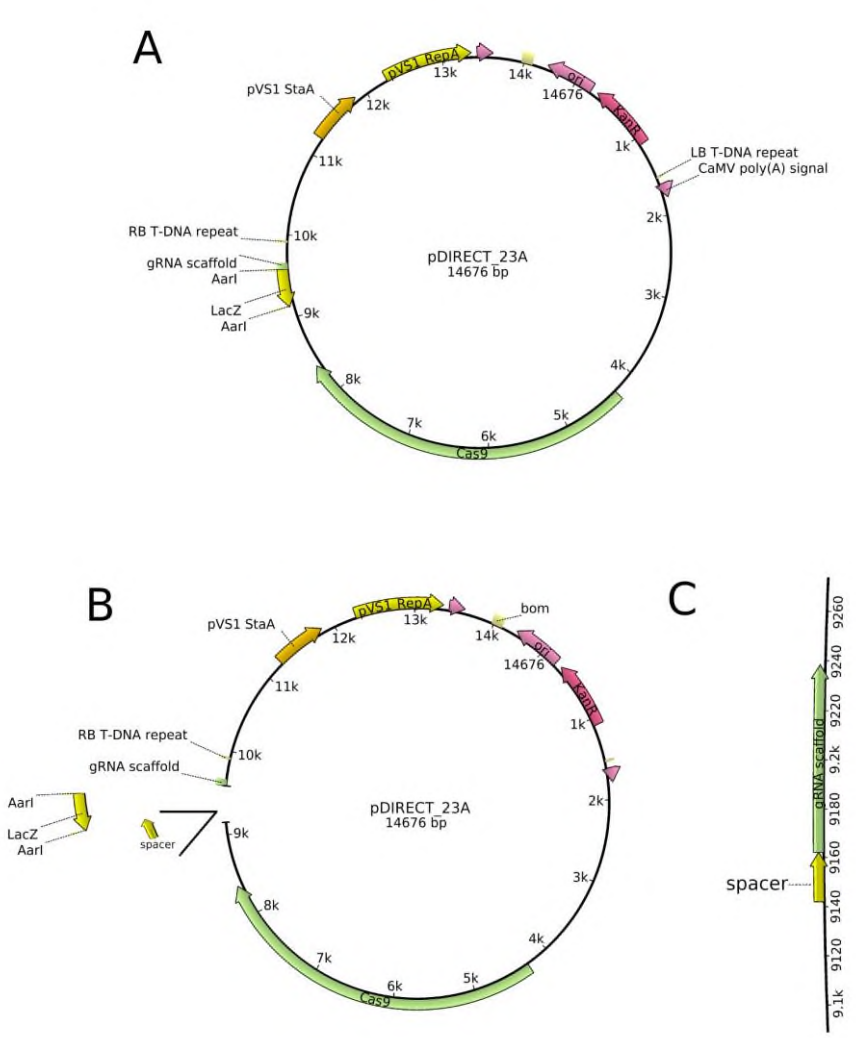

Fig. 1. Cloning of the gRNA spacer into the pDIRECT_23A vector. A – intact vector, B – cloning of the gRNA spacer instead of the LacZ gene, C –part of the resulting genetic construct.

2.4 Agroinfiltration

Plasmids were introduced to *A. tumefaciens* by electroporation on a MicroPulser (Bio-rad). Bacterial clones containing the plasmids were selected on LB medium supplemented with 150 mg/l rifampicin and 100 mg/l kanamycin. The presence of the plasmid was verified by PCR with the primer GGAATAAGGGCGACACGGAAATG as forward, and gRNA spacer as reverse primer.

Selected clones were grown on solid LB medium supplemented with antibiotics for 24h and then suspended in the infiltration medium (10 mM MES pH5.5, 10 mM $MgCl_2$, 150 μM acetosyringone, pH 5.5) to OD 600 of 0.75, according to Mooney and Graciet [17]. The abaxial side of the leaves was scratched with a syringe needle to promote infiltration. Bacterial suspension was infiltrated into these areas with a blunt 1-ml syringe. Three plants were used to test each genetic construct. Tissues were analyzed visually 3-5 days later.

3 Results

3.1 gRNA spacer design and plasmid construction

All studied genes are presented in *A. thaliana* by one copy. However, these genes are not annotated in *B. napus* and their functions have never been studied. With regard to the facts that *B. napus* is a tetraploid, there might be several copies of each gene. It is known that duplicated genes sometimes loose their functions or develop new ones. There are 12 known orthologs of *AtMYB60* gene in 8 other *Brassicaceae* species. Only one copy can be found in A8 chomosome of *B. napus* by sequence homology via BLAST. Four copies of *MYBL2* gene are present in the genome of *B. napus*, and there are also 13 known orthologs of this gene in 9 other *Brassicaceae* species. There are four transcript variants of *CPC* gene in *B. napus* and 17 known orthologs of *AtCPC* gene in 10 other *Brassicaceae* species. gRNAs were chosen to target the most conserved regions of these genes, that are present not only in *B. napus*, but also in the ancestor species *B. rapa* and *B. oleracea*. The variety 'Ratnik' is of local breeding, and its genome have never been sequenced.

However the CRISPOR software predicted a lot of possible guide sequences, only few of them fell within the most conserved regions and could be used in this study. Finally, two gRNA spacers were synthesized to target *MYB60* and *CPC* genes, and only one – for *MYBL2* gene. List of the oligonucleotide sequences are presented in the table 1.

Table 1. gRNA spacers for the knockout of the target genes.

gRNA spacer	gRNA spacer + PAM
MYBL2	GTGATGGTAGAGACGATGAT TGG
MYB60 (1)	GACAAGATAGGGATCAAGAA AGG
MYB60 (2)	GTGGTAGATATGAAGCTA TGG
CPC (1)	GATATTGGCTTATGAAACA CGG
CPC (2)	GGTGGGAATTGATAGCCGGA AGG

All designed gRNA spacers were successfully cloned in the vector pDIRECT_23A. Sequencing showed that synthesized oligonucleotides were inserted in the right place and direction, as expected. Direct transformation vector, engineered by Čermák et al. [6], proved to be time-saving and easy tool for genome editing in plants.

3.2 Agroinfiltration

The effect of the expression of the genetic constructs was already visible on the third day after agroinfiltration. However only the abaxial side of the leaves was treated with *A. tumefaciens*, visible anthocyanin pigmentation was observed on the both sides. Pigmentation was the most intense near the scratches and in the leaf veins. Five-seven days after agroinfiltration anthocyanins spread all over the leaf.

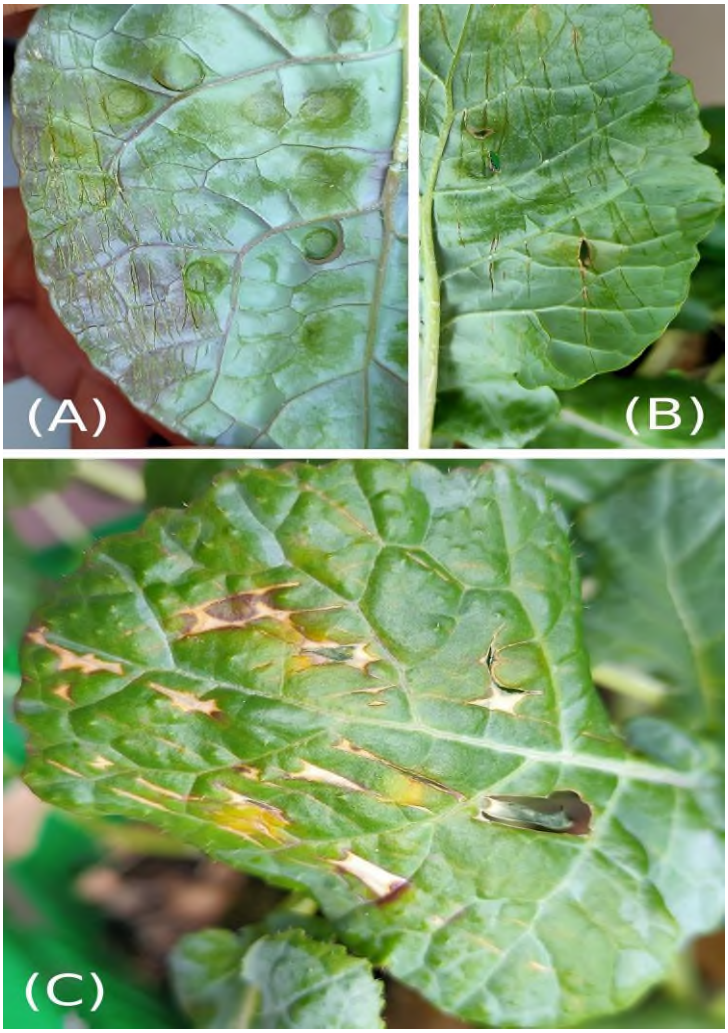

Fig. 2. Leaves of *B. napus* after agroinfiltration with genetic construct: A - MYB60 (2); B – CPC (1); CPC (2).

The second gRNA spacer targeting *MYB60* gene was the most effective (table 1, fig. 2A), as well as the second gRNA spacer targeting *CPC* gene (table 1, fig. 2C). Treatment of the leaves with the plasmid targeting the *MYBL2* gene resulted in the less intensive pigmentation. However, the first gRNA spacers for both *MYB60* and *CPC* genes (table 1, fig. 2B) did not promote any changes in color in three days.

It is to note that in five-seven days after agroinfiltration, chlorosis was observed in older leaves, regardless of the genetic construct, probably due to *Agrobacterium* infection [18]. Older leaves were more susceptible to agroinfiltration than younger leaves.

4 Discussion

It was demonstrated that each of the targeted genes regulate anthocyanin pigmentation in *B. napus*. However, the efficiency of the chosen gRNA spacers varied. MYB60 (2) and CPC (2) spacers (table 1) promoted the most intense anthocyanin pigmentation. These two spacers interfere within DNA-binding domains of MYB60 and CPC transcription factors, however CPC (1) spacer hits this region only partially. The target of MYB60 (1) spacer is located in the beginning of the R2R3 conserved domain, which does not interact with DNA. MYBL2 spacer also doesn't hit the DNA-binding domain. It can explain the lower intensity of the anthocyanin pigmentation in the infiltrated leaves.

It is to note that it is not easy to perform agroinfiltration on *B. napus*, because the effect strongly depends on the leaf development stage, the thickness of the lower epidermis and occurrence of the leaf veins. Diffusion of bacteria by the syringe pressure was more problematic in younger leaves due lo the high density and projection of the veins. Therefore effects of the expression of the genetic construct was observed mostly locally. Older leaves appear to be better objects for such experiments. The problem of chlorosis can probably be solved by lower concentration of bacteria.

It is of great interest and scientific novelty to knockout *MYB60*, *CPC* and *MYBL2* genes of *B. napus* by genome editing. Resulting plants can obtain not only increased anthocyanin pigmentation, but also increased stress tolerance. MYB are the most common transcription factors described in plants, contributing to plant defense against stress. *MYB60* [12] belongs to R2R3 subfamily and include DNA-binding domain and an activation/repression domain [19]. A total of 126 R2R3-MYB members have been identified in *Arabidopsis thaliana*. They are involved in the regulation of metabolism, cell fate determination, growth and development, tolerance to drought and salinity stress and different interactions.

There are only six single-repeat R3 MYB transcription factors in the *Arabidopsis* genome. They are involved in developmental processes such as root hair differentiation, stomatal formation and trichome initiation. CPC [13] and MYBL2 [14] are among these factors. Thus, plants with lower content of CPC and MYBL2 could develop less stomates and therefore increase drought tolerance [20].

5 Conclusions

The possibility to increase anthocyanin pigmentation by the knockout of *MYB60*, *CPC* and *MYBL2* genes of *B. napus* was demonstrated in this study. gRNA spacers targeting DNA-binding domains of transcription factors encoded by these genes proved to be the most effective. Genetic constructs created within the framework of this research can be used for *in vitro* or *in planta* transformation of *B. napus.*

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