MsDwf1^{-} dwarf plants of diploid *M. sativa* carry a mutation in the gibberellin 3-β-hydroxylase gene

Ph. D. Thesis

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Introduction

Alfalfa (*Medicago sativa*) species are world wide spread economically important plants of the Fabaceae family. *Medicago* species capable of symbiotic interactions with *Rhizobia* (nitrogen fixation) and endomycorrhiza fungi (phosphorus acquisition) are in the focus of recent molecular biology studies. In addition to symbiosis, the understanding of pathogenic interactions and other plant biological pathways in *Medicago* is tremendously helped by the results and resources of the advanced *Medicago truncatula* genome programs (www.medicago.org). Demonstrated macro- and microsynteny between the genomes of the model legume *M. truncatula* and other related species like diploid and tetraploid *Medicago sativa* (Choi et al., 2004) and pea (*Pisum sativum*) (Kaló et al., 2004) makes these plants easy targets to reveal gene functions behind natural or induced phenotypes. The utilization of the results obtained obligately on *M. truncatula* is hardened by the incompatibility to produce fertile progeny of *M. truncatula* and *M. sativa*.

Diploid alfalfa subspecies are in closer relationship with the harvested tetraploid alfalfa than *M. truncatula*. Alfalfa plants are out-crossing; consequently (most if not all of) the genome is in heterologous configuration. As a result, different mutant alleles can be maintained in the population, and selfpollinated progenies can generate mutant phenotypes when naturally occurring mutant alleles become homozygous. This phenomenon was utilized by Kiss et al. (1993) during the generation of the diploid *M. sativa* gene map. In this study, one of the phenotypes observed in the mapping population of the above-mentioned work, the dwarf growth is investigated. To be more effective we followed more than one experimental strategies during the isolation of the gene responsible for the phenotype: once the genetic region determining the MsDwf1$^-$ phenotype was dissected by map based cloning approach; on the other hand candidate gene - pinpointed based on the mutant phenotype - was selected.

To carry out this work several tools, as the detailed genetic map of diploid *M. sativa*, the mapping population belonging to the map, and for the physical mapping the *M. truncatula* BAC library were available in the workgroup. The candidate gene was pinpointed based on the similarity of MsDwf1$^-$ to mutant phenotypes of other plants, which were dissected in previous works and the corresponding mutant gene was isolated before. At the end a pea mutant leaded us to the proper candidate gene. In the following experiments we proved that the candidate gene is identical with the gene of interest, whose mutation caused the dwarf phenotype of the diploid alfalfa plants.

Materials and methods

Plant material

F2 mapping population was originated from the cross between two diploid subspecies of alfalfa, *M. sativa ssp. quasifalcata* (Mqk93) and *M. sativa ssp. coerulea* (Mcw2) (Kiss et al., 1993). Plants were maintained in pots under greenhouse conditions. Plants from the extended population were grown in SANYO MLR-350 growth chambers under long day light condition to the end of the primer testing. Paternal alleles were sequenced from homozygous plants originating from the self-crossed Mcw2 population. For the heterologous transformation experiment the mutant *ga4 Arabidopsis* stock of was ordered from Nottingham Arabidopsis Stock Centre. T1 seeds were sown under sterile conditions onto ½ MS medium contaning 15 µg/ml kanamycin (Duchefa). T2 plants were also grown in MLR-350 Sanyo growth chambers under long day light condition in 5 cm diameter pots.

Physiological tests

Cuttings of two wild type and two dwarf individuals from the segregation population were investigated in gibberellin tests. Bioactive GA_3 (Sigma, St.Louis, MO) has been dissolved in ethanol and diluted with water. 100 ml of bioactive GA_3 solutions in a final concentration of 70 or 140 µM were applied to the soil of the plants grown in 15 cm diameter pots four times in a two-week period. The length of the third internode of the shoots was measured three weeks following the last GA_3 treatment. Grafting experiments were carried out according to the previously described method (Krusell et al., 2002). Growth habit was monitored for additional six months.

DNA handling methods

Total DNA to the amplification was isolated with ZenoGene Kit, to hybridization with the CTAB method (Doyle and Doyle, 1987). PCR (Polimerase Chain Reaction) experiments were carried out using the buffer described by Sambrook and Russel (2001), the final concentration of $MgCl_2$ was 1,5 mM. Amplifications were performed in MJ Research PTC-200 or ABI GeneAmp PCR Systems 2700 thermal cyclers. PCR conditions were optimized for each primer pairs. Primer pairs were designed using Primer Premier and Primer Select. Genetic mapping and analysis was performed according to the color-mapping method described by Kiss et al. (1998). Markers showing no polymorphism in length were first genotyped with SSCP (Single-Strand Conformation Polymorphism) as described previously by Orita et al. (1989). Samples were mixed with STOP dye in an equal proportion and boiled in order to denaturate on 98 ºC for five minutes. Polymorphic bands were separated in native

polyacrylamid gels. Beside SSCP single nucleotide polymorphisms were also detected with heteroduplex analysis following *Cel*I digestion (Oleykowski et al., 1998). Prior to *Cel*I digestion heteroduplexes were formed from amplificatums, and following a nuclease reaction fragments were separated on denaturing polyacrylamid gels. Polymorphic bands were visually analyzed following silver staining in both SSCP and heteroduplex analysis.

Methods described by Sambrook and Russel (2001) were applied with minor modifications in hybridzation experiments. Fragments were labelled using Amersham Biosciences, Ready-To-Go DNA Labelling Beads (-dCTP), which incorporates α-[^{32}P]dCTP. Hybridization signal was visualized by phosphorimage analyzer (Amersham Biosciences), using a Storm 840 (Molecular Dynamics) instrument.

Working with BAC clones and genome walking

The second BAC library (mth2) was used to create the Dwf1 contig. BAC clones of the contig were primarily identified with bioinformatic tools and multiplex PCR reactions. The *Hin*dIII fingerprint data of *M. truncatula* BAC clones (www.medicago.org/genome) were also used to the construction of the Dwf1 contig. BAC DNA of the selected clones was isolated with a slightly modified alkaline method described by Sambrook and Russel (2001).

Allele sequencing, cloning of PCR fragments

Parental alleles (Mqk93 and Mcw2) for *GA3ox* gene were amplified from total DNA of homozygous plants of the F2 mapping and self-crossed Mcw2 population using *M. truncatula*-based primer pairs. Amplified fragments were purified with GFX columns (Amersham) and ligated into pGEM-T Easy (Promega) vector, and transformed into *E. coli* XL1Blue competent cells. Plasmid DNA was isolated from the transformant colonies according to Sambrook and Russel (1982).

***Arabidopsis* transformation**

Primarily *E. coli* DH5α strain was used to create plasmid constructs, then they were transformed into *Agrobacterium tumefaciens* GV3101 strain with conjugation using S17 strain. The fragment of 7K2 BAC clone was ligated into pCambia2201 binary vector (www.cambia.org). This vector containes npt_{II} gene which confers kanamycin resistancy as plant selection marker. MsLehi construction was created from MtLe plasmid. In this plasmid the main body of the structure gene originated from the MsDwf1^{-} plant. Plant transformation was done with „floral dip" method as described by Clough and Bent (1998).

RNA methods

Total RNA was prepared from *Arabidopsis* shoots as described by Szittya et al. (2002). RNA concentration was measured with NanoDrop equipment. cDNA was synthetised with

Roche Transcriptor High Fidelity cDNA Synthesis Kit according to the instruction of the manufacturer in 10 µl reaction volume.

Thesis

1. Mutant dwarf plants differ from the wild type only in their shoots. We could not experience other apparent morfological differences compared to the wild type. This resembles to the characteristic features of the phenotypes described as "semidwarf" in previous works. Self-crossed progeny analysis of the parents of the mapping population revealed that the paternal parent (Mcw2) carried the dwarf phenotype. Treatments with exogenous GA_3 solutions effectively restored the growth of mutant plants to normal wild type phenotype. On the other hand complementation was not experienced when mutant shoot was grafted onto wild type stock.

2. To determine the genetic position of the *Msdwf1* gene precisely, the mapping population was extended in two steps. Genotyping effort of 317 plants resulted in the positioning of the *Msdwf1* gene between markers L295 and OPG4AB. These markers were then used to screen additional individuals to identify more plants with recombination event between these two flanking markers, and these plants were used to create a more accurate gene map. Based on mapping data of 1031 individuals U189 was found to be in closer position, and CycMs3 turned out to be tightly linked by zero predicted recombination to MsDwf1$^-$ phenotype.

3. With the sequence information of markers tightly linked to MsDwf1 locus chromosomal walking was started using the clones of the second *M. truncatula* BAC library (mth2). Overlapping clones were identified to create a contig spanning the chromosome region between the two closest flanking markers. CycMs3 and U189, as the tightliest linked markers, were used to identify the first BAC clones. Primers generated from the newly identified BAC clones were used to genetically re-map them. These efforts resulted in the delimiting the genomic region containing the putative *Msdwf1* gene between U189 (~0,1 cM from MsDwf1) and 22D10_1 (~0,2 cM from MsDwf1) markers.

4. Candidate gene was searched for based on the characteristics of the phenotype. To see whether the gibberellin 3-β-hydroxilase gene (*GA3ox*) mutated in the MsDwf1$^-$ plants *Medicago* orthologs of the gene were identified. BLASTn search was used to identify the *M.*

truncatula TC97820 showing homology to the pea *Le* gene, and a sequenced *M. truncatula* BAC clone mth2-7K2 (AC144340) from the NCBI GeneBank. The map position of the *GA3ox* gene was determined on the *M. sativa* population with the help of a specific intron-spanning primer pair (MsLe). Genetic mapping confirmed absolute co-segregation of the MsLe marker with the locus of the dwarfism, and BAC mth2-7K2 could be inserted into the *M. truncatula* BAC contig we created.

5. We presumed that MsDwf1$^-$ plants carry such a mutation in *GA3ox* gene, which is probably deleterious for enzyme activity. In order to prove our hypothesis the wild type and the mutant paternal alleles from the heterozygous male parent (Mcw2), as well as the wild type maternal allele (Mqk93) from the population were amplified, cloned and sequenced with the help of *MtLe*-based primer pairs. The protein sequence of the alleles of Mcw2 plant differ only in the Gly126Asp position. The Mqk93 allele has, similarly to the wild type Mcw2 allele, glycine in the 126. position.

6. Phylogenetic analysis was done with the amino acid sequences originating from different species in order to position the GA3ox of *Medicago sativa* among the genes, which constitute small gene families in several species. According to their evolutionary nearness the isolated *M. sativa* gene shows the greatest similarity with *Mtle* (97%) and pea *Le* (gibberellin 3-β-hydroxilase; 89%) in the protein level.

7. In order to determine the copy number of *GA3ox* gene DNA-DNA hybridization was performed. A single strong signal was observed for *M. truncatula* A17. In the lane of *M. sativa* plants of the mapping population in case of *Dra*I and *EcoR*I RFLP polymorfism was detected, which co-segregated with the dwarf phenotype. PCR experiments also supported the presumption, that *GA3ox* is a single copy gene in the two *Medicago* species. We did not succeed to find candidates for the putative homologs of other GA3ox enzyms with blast-based homology searches using the *Arabidopsis thaliana GA3ox* genes.

8. *Arabidopsis* heterologous transformation experiments were performed to compare constructs containing wild type *Medicago truncatula GA3ox* gene (MtLe) and the structure gene of Mcw2 mutant allele originating from the MsDwf1$^-$ plant (MsLehi). Both constructs were transformed into *Arabidopsis ga4* mutant plants with *Agrobacterium tumefaciens* transformation system. Transformed plants containing the MtLe construct had a similar

appearance as the wild type Landsberg erecta plants. In contrary we did not detect significant difference in the height of MsLehi transformants compared to the dwarf mutant *ga4* plants. mRNA of the transgene was demonstrated in reverse transcription experiment of transgenic plants.

Conclusions

In our experiments the MsDwf1$^-$ „semidwarf" phenotype could be reverted with exogenous GA3 treatment. From this we concluded that one of the genes of GA biosynthesis enzymes mutated, and as a reason the GA level possibly decreased resulting the dwarf phenotype. Following the grafting experiment it was plausible to suppose, that in the wild type shoot the bioactive endproduct (GA_1) of the GA biosynthesis is non-transportable, and, on the other hand, in the mutant shoot the transportable precursors can not be converted to bioactive derivatives. Our findings were reminiscent of the results of previous works done on the pea *le* mutant, so it was plausible to suppose that the final step of the GA_1 biosynthesis is affected in the investigated dwarf mutant. In pea *le* plants Mendel's dwarf phenotype was a consequence of a mutation in the gibberellin 3-β-hydroxylase (*GA3ox*) gene.

This pheotype was mapped previously several times with the help of relatively small mapping populations, and the corresponding locus was placed on LGIII in the pea genetic map (Weeden et al., 1998). This work describes the fine mapping of *MsDwf1* gene in an extended population, where a single locus was defined for the phenotype. We confirmed that the gene labelled with the MsLe molecular marker coincide in genetic and physical way with the *MsDwf1* locus of the phenotype. Since Msdwf1 genomic region and pea region containing *GA3ox* (*Le*) gene are synthenic (Kaló et al., 2004), and the nucleotic sequence of *M. truncatula* and the pea gene show great similarity, this gene of Medicagoes was considered to the ortolog of the pea *Le* gene.

Mqk93 and Mcw2 (*MsDwf1* and *msdwf1*) alleles were sequenced. The *msdwf1* allele has an amino acid change in the Gly126Asp position. Since the GYG motif is highly conserved in the protein family, Gly126 could have an important role in the enzyme, and its mutation could decrease or diminish the enzyme activity. This is the only amino acid alteration between the wild type (*MsDWF1*), and mutant (*Msdwf1*) alleles of Mcw2 plant. Taking all this into consderation, we suppose that this mutation is responsible for the MsDwf1$^-$ phenotype. Other alterations between the Mcw2 and Mqk93 alleles probably do not disturb the function of the enzyme, and can be regarded as natural allelic variants of the wild

type gene. The Gly126Asp change occured relatively far from the known motifs of 2-oxoglutarate (2OG) and Fe(II)-dependent oxigenase protein family. Although Gly126 is highly conserved in ortologs of plant species and distant members of the protein family as well, the function of amino acid residue corresponding to Gly126 in MsDWF1 has not been determined yet. So, these dwarf plantsand the isolated mutant *GA3ox* alleles provide opportunity to find out more from the functioning of the enzyme.

PCR and hybridisation experiments could only detect one copy of the *M. truncatula GA3ox* gene. On the other hand we could not exclude that *GA3ox* genes with relatively divergent nucleotide sequence but coding enzymes with similar enzyme activity exist in the genome of *Medicagoes*. Moreover this hypothesis was supported by the „semidwarf" feature of the phenotype. In *Arabidopsis thaliana* enzymes of the GA3ox family have mainly distinct partly overlapping roles (Mitchum et al., 2006). Our attempt to find ortologs of other *Arabidopsis GA3ox* genes in *M. truncatula* with bioinformatic tools was unsuccessful. This, however, does not mean that these genes do not exist, since the genomic sequence of *M. trucatula* is not complete yet, and sequence similarity between the two species could be insufficient to allow the identification of these genes.

The results of the transformation experiment carried out on *Arabidopsis ga4* mutant plants coincide with our previous hypothesis, that the investgated *GA3ox* gene has undergone such a mutation in the MsDwf1$^-$ plants, which caused the dwarfism.

Present thesis is about a mutation in a *GA3ox* gene. In case of *Medicago* species this gene, its mutant allele and the corresponding phenotype was not described prior to our publication. The gene was neither found in the database of Flanking Sequence Tags-FSTs of more than 17 000 T-DNA mutant lines produced by the Noble Fundation. Consequently MsDwf1$^-$ plants could be the key for further studies of *GA3ox* proteins originating from the *Medicago* species.

According to previous comparative mapping studies high degree of similarity was found in the investigated genomic region of *M. truncatula* and *M. sativa* in our work. Our results concide with that one, which were described before in the literature of comparative genomics of *Medicago* and *Pisum* species.

The identification of the *MsDWF1* gene is another example of the successful combination of using genomic resources developed for a model plant species (*M. truncatula*) and the accomplishments of pea genetic studies, and unravelling a biological problem with map based cloning methods in a crop plant (*M. sativa*). Besides the identification of the *Msdwf1* gene there are other achievements of the work described in this thesis; we created a

contig of overlapping genomic BAC clones, which could facilitate the purposive, economical sequencing of this region of the *M. truncatula* second linkage group. In view of the whole sequence of this genomic part potentially other agronomically or from other point of view interesting genes mapped in this region could be isolated.

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