MOLBI: 129119 záró jelentés (harmadik év/third year).

PD129119 - Defining the targeting preference of SlDML2 DNA demethylase to ripening genes in tomato (A termésérés szabályozásának epigenetikai háttere paradicsomban).

Background

DNA methylation is a major and conserved epigenetic mark that is involved in many biological processes. In plants DNA methylation can occur at cytosine (C) both in symmetrical (CG or CHG) and asymmetrical (CHH) contexts and is controlled by three classes of DNA methyltransferases (1,2). Plants can also actively demethylate DNA by 5'-methylcytosine DNA glycosylase/lyase enzymes, including DEMETER-Like DNA demethylases (DMLs), that remove methylated cytosine and replace by a nonmethylated cytosine (3). The tomato genome contains four putative DNA demethylases (SlDML1, SlDML2, SlDML3, SlDML4) (4). While DNA methylation is known to inhibit gene transcription by forming compact, inactive chromatin, demethylation has anti-silencing effect leading to hypomethylation of sites important for DNA–protein interaction thus inducing gene expression (3,4). The epigenome of an organism is not static, it changes during development and in response to environmental signals. A dynamic interplay of methylation and demethylation defines the gene expression pattern of cells (1,2). Genome-wide methylation/demethylation waves are essential for epigenetic reprogramming during eukaryotic development (4). This epigenetic reprogramming establish and maintain different gene expression patterns to ensure proper cell differentiation thus cells with identical genetic material become different types of specialized cells with specific function and structure (4).

Fruit ripening is a complex developmental process that is under strict hormonal, genetic and epigenetic control. SlDML2-mediated active DNA demethylation is a major determinant of tomato fruit ripening. During ripening, tomato fruits undergo a global DNA demethylation due to the increased expression of SlDML2 (5). It activates genes, involved in fruit ripening and silences genes involved in fruit growth that become unnecessary during ripening (1). This regulation represents an elegant and unique epigenetic reprogramming as it can switch the function of fruit pericarp cells from growth to ripening, without inducing cell differentiation. The molecular mechanism behind – how SlDML2 is preferentially targeted to genes relevant to fruit ripening – and the regulation of SlDML2-mediated DNA demethylation has not been studied in any crop plants to date. In Arabidopsis, the target selection of the DNA demethylases is defined by IDM complex (2) (Fig. 1A). MBD7 (canonical Methyl-CpG-binding domain) and HDP2 (Harbringer transposon derived DNA methyl-binding-protein) two components of IDM complex, ensure that the IDM complex is targeted only to highly methylated regulatory sequences, where active DNA demethylation is required for expression (2). IDM2 and IDM3 proteins function to connect the DNA recognition components to the core enzyme, IDM1 histone acetyltransferase. In addition, IDM2 and IDM3 also function as chaperone proteins to ensure IDM1 activity (1). They belong to the family of α-crystallin domain proteins, most of which are small heat shock proteins that function as protein chaperones (2). IDM1 create a permissible chromatin environment for ROS1 reqruitment and function by specifically binding to chromatin sites that lack histone H3K4 di- or trimethylation and acetylating H3 (2). 18 putative tomato MBD proteins (SlMBD) were recently identified using in silico experiments (3) and we could also reveal the existence of putative IDM1, IDM3, HDP2 tomato proteins (SlIDM1, SlIDM3, SlHDP2). These data suggested that DNA demethylation by SlDML2 in tomato fruits might act in a similar manner like in Arabidopsis. Despite of its essential role in fruit

ripening the molecular background of SlDML2 demethylation and target preference is highly unknown. Understanding the target selection of tomato SlDML2 could help us to gain insight into the epigenetic reprogramming of differenciated cells and it could serve as an important tool to regulate tomato fruit quality. In the first part of the project, we generated transgenic MoneyMaker tomato plants from the respective SlMBD, SlHDP and SlIDM candidates by using CRISPR/Cas9-driven mutagenesis to reveal the involvement of these protein factors in SlDML2 target selection during fruit ripening.

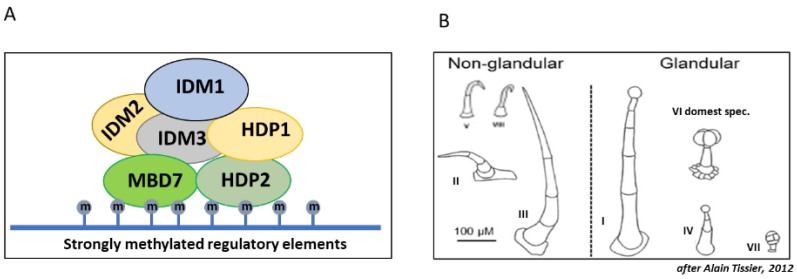


Figure 1: A. The structure of Arabidopsis IDM complex. B. Different trichome types in tomato.

In addition to its importance in fruit ripening studies, tomato is also the model plant for the studies of glandular trichome development. Studies of trichome formation have focused on the model plant Arabidopsis, that produces one type of unicellular, non-glandular trichome. Tomato has eight different trichome types, including both glandular and non-glandular multicellular trichomes (Fig. 1B) (6). They serve as physical and chemical barriers against herbivores. Glandular trichomes synthesize and store a wide range of specialized metabolites, such as terpenoids, phenylpropanoids, flavonoids, alkaloids, and acyl sugars (7). In tomato, the focus of research has been on glandular trichome development and the metabolites they secrete (8, 9). A couple of different transcription factors involved in glandular trichome initiation and morphogenesis have recently been identified (6). Woolly (Wo) is an HD-ZIP IV transcriptional factor, regulating type-I glandular trichome formation (10). CyclinB2 (SlCycB2) is induced by Wo and participated in Wo-mediated type I trichome development (10,11). The C2H2 zinc-finger protein, Hair (12) gene interacts with Wo and it is also essential for type I trichome development (11), while the bHLH protein 4 / 48 MYELOCYTOMATOSIS-RELATED 1 (SlMYC1) is essential for type VI glandular trichome development. (13, 14). Hairless mutant is, characterised by a distorted morphology of type-I trichomes and a deficient accumulation of sesquiterpenes in type-VI trichomes (9). HAIRLESS codes for SRA1, a subunit of the SCAR/WAVE multiprotein complex that controls actin filament nucleation and polymerization (9). Another tomato mutant, hair absent, which exhibits a complete absence of type-I trichomes on the epidermis, has also been characterised, and encodes a C2H2 zinc-finger protein. SlMIXTA-LIKE acts as a general repressor of trichome formation (15). SUVH3, histone lysine methyltransferase mutants (hairplus) exhibit high type-I trichome density suggesting for the first time the implication of the epigenome in the control of glandular trichome formation (16). All these findings demonstrate a very different regulatory pathways and protein complexes in tomato compared to which regulates unicellular trichome initiation in Arabidopsis. Although the majority of flowering plants produce multicellular trichomes our knowledge about the molecular mechanism of multicellular trichome formation is still very limited. Furthermore, the importance of epigenetic control in glandular trichome development in crops is poorly understood. Beside its role in fruit ripening, the involvement of SlDML2 DNA demethylation in other biological processes remains to be unravelled. Indeed, the role of tomato IDM complex in

any biological pathways has not been studied yet. Based on our preliminary results (see below in part 2. of results) idm3 and idm1 mutant CRISPR lines show increased glandular trichome density. Thus in the second part of the project, we conducted various transient and transgenic assays to experimentally demonstrate the involvement of IDM complex and DNA demethylation in glandular trichome development.

Results

1) **<u>IDM complex proteins have no important role in DML2-mediated fruit ripening in tomato</u>**

During the first part of the project we continued transient virus induced gene silencing (VIGS) experiments of SlMBD, SlHDP and SlIDM genes, to rapidly preselect the best candidates before transgenic CRISPR/Cas9 targeting. The effect of SlDML2 VIGS were previously described, resulting mottled fruits with red (non-silenced-ripened) and yellow (silenced-unripened) sectors (17). We expected that if our SlMBDs and/or SlIDM candidates are involved in fruit ripening than similar to the positive controls, yellow areas in the VIGS treated fruits will indicate the inhibition of ripening. We applied two VIGS technique: sprout vacuum infiltration and injection into the carpopodium of mature tomato fruit, however the efficiency of VIGS silencing of the respective genes was inefficient in both case (validated by qPCR) and we could not detect the inhibition of ripening. Therefore, I decided to close VIGS experiments and focus on the generation/characterization of transgenic SlMBD, SlHDP and SlIDM CRISPR/CAS9 lines. To generate the respective knockout mutants, two gene-specific CRISPR guide were designed for each gene using the online CRISPR 2.0 program. We have generated 8 CRISPR/CAS9 mutant tomato lines, 2 independent CRISPR lines (guide1 and guide2) from each genes (MBD7, IDM1, IDM3, HDP2) to avoid off target effects. SlMBD, SlIDM and SlHDP factors were preselected based on their fruit specific expression (by qPCR screen) (Fig. 2).

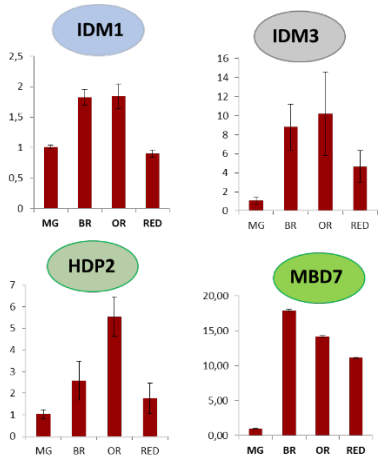


Figure 2: The level of putative tomato IDM complex components are increased during fruit ripening. qPCR measurement of mRNA levels from WT tomato fruits from different developmental stadium. MG-mature green stadium of tomato fruit, BR-breaker stadium, OR-orange stadium, RED- red ripening stadium. Mean values were calculated from five independent samples (n = 5).

CRISPR/CAS9 targeting induced distinct deletions (1 to 5 nt deletions) and short (1 nt) insertions leading to formation of aberrant mRNAs harboring premature stop codon (PTC) close to the CRISPR target site (in the 1st or 2nd exon) (Table1).

Gene Name	Gene ID	Mutations in the first exon
MBD7	Solyc06g068140	Deletion, insertion
IDM1	Solyc07g062600	Deletions, insertions,
HDP2	Solyc08g069160	Deletions, Transition (C to T)
IDM3	Solyc04g082720	Deletions, Insertions

Table 1: The list of generated CRISPR/Cas9 lines

Accordingly, our qPCR measurements show that the mRNA level of the targeted genes are significantly reduced in the respective CRISPR/Cas9 lines. However, inhibition/delay of ripening could not be detected in T1 homozygous lines suggesting that IDM complex proteins are not dispensable for DML2-mediated fruit ripening. In Arabidopsis, IDM complex only recruite to a subset of genomic regions demethylated by ROS1 DNA demethylase, thus additional mechanisms for directing ROS1 for locus-specific demethylation must exist (1-3). We suppose that multiple ways of DML2 targeting, independent from IDM complex, might also exist in tomato. Interestingly mbd7 CRISPR/CAS9 lines show an unexpected multilocule fruit shape phenotype and it can also affect the compoundness of the leafs (the number of leaflets are increased) (Fig. 3).

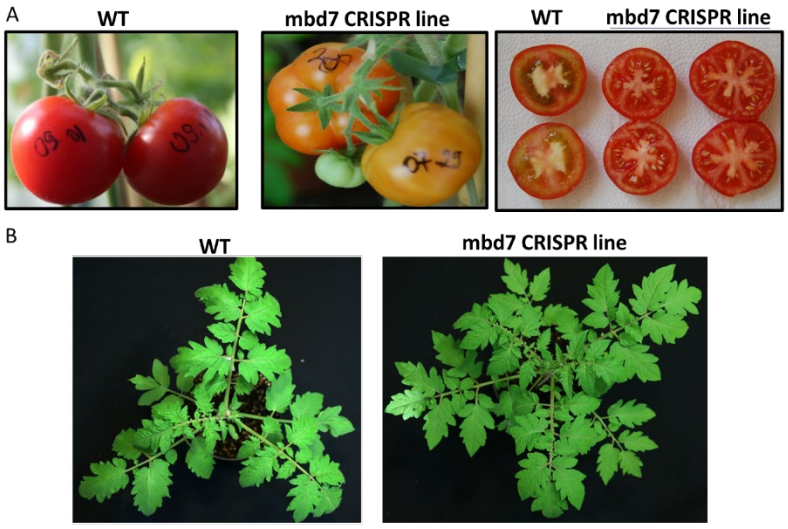


Figure 3: A. MoneyMaker tomato variety produces a round medium size fruit with two locule inside. Fruits on mbd7 CRISPR lines grow bigger and shows multilocule phenotype. B. WT plants with normal compound leaves. mbd7 CRISPR lines with abnormal, exccessively divided leaves.

Genes that were identified to be responsible for these multilocule fruit and/or compoundness leaf phenotype in tomato are all involved in CLAVATA-WUSCHEL signaling pathway (18). CLAVATA-WUSCHEL signaling are known to be involved in meristem stem cell maintenance (19). We will use mbd7 (Methyl-CpG-binding domain protein) CRISPR/CAS9 line in the future to unveil how DNA demethylation affect meristem size in tomato.

2) IDM3 α-crystallin domain protein has role in glandular trichome development

2.1 **IDM3 mutation enhance glandular trichome density**

In the second part of the project, we have analysed idm3 (INCREASED DNA METHYLATION 3, Solyc04g082720) CRISPR lines that show an unexpected, conspicuous phenotype with significantly higher trichome density on tomato leaves, stems and inflorescence (but not in roots and fruits) (Fig. 4A).

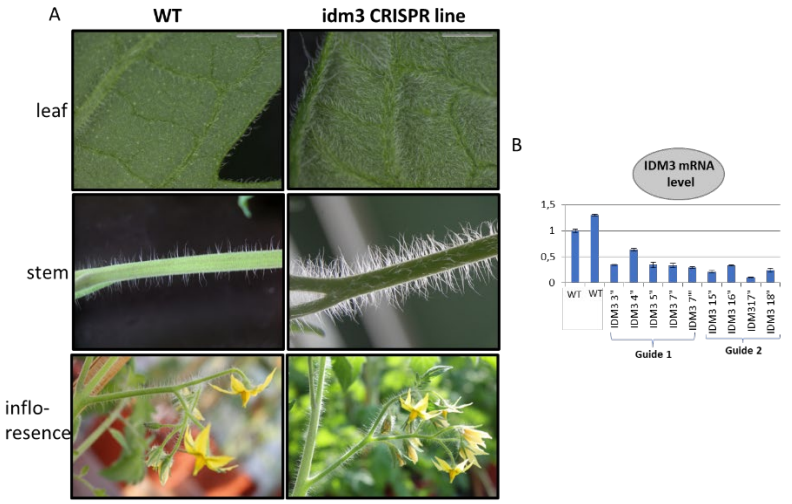


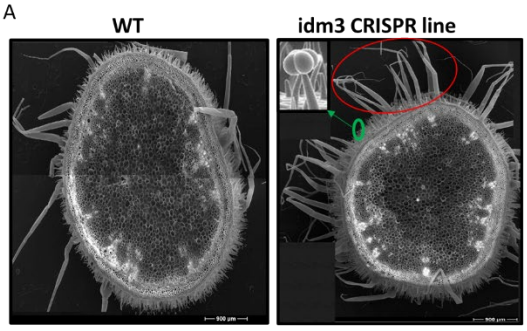
Figure 4: A. Trichome density of leaves, stem and infloresence in idm3 CRISPR lines are higher compared to the WT. B. The mRNA level of IDM3 is decreased in idm3 CRISPR lines.

The Arabidopsis orthologue of IDM3, is a chaperone protein, that belongs to the family of HSP20 α-crystallin domain proteins. It is a component of the IDM complex and it prevents DNA hypermethylation through ROS1-mediated DNA demethylation pathway in Arabidopsis (1). Tomato IDM3 harbours similar domain structure to its Arabidopsis homolog thus it may also function as a chaperone to ensure the assembly of tomato IDM complex. Since the epigenetic aspects of glandular trichome development were not studied yet, we decided to investigate in detail how IDM3 could affect the density of glandular trichomes.

We have used CRISPR-Cas9 genome editing system with two independent guides (sgRNA: guide 1 and 2) to specifically target the first exon of tomato IDM3. We selected two independent T2 homozygous lines, each harbouring a premature stop codon in the first exon. Slidm3-1 (guide1: 1 nt deletions, that results an early stop codon 153 nt after the start codon) and Slidm3-2 (guide2: 4 nt deletion, that also results an early stop codon 51 nt after the start codon). The coding sequence of IDM3 is 474 nt long.The IDM3 transcript level was strongly decreased in these mutant lines perhaps due to the activity of Nonsense Mediated mRNA Decay (Fig.4B). idm3 lines (guide1 and guide 2) showed a very similar phenotype and results thus I will show you only the results of idm3 guide 1 line (called idm3 CRISPR line hereafter).

In order to determine the trichome types characterisitic to idm3 mutant lines, trichome identity and density of WT and idm3 mutant plants were analysed by scanning electron microscopy (SEM). SEM comparison of tomato leaves and stems show that relative to wild-type, idm3 CRISPR lines display a significant increase in the density of I and VI glandular trichome types (3-4 times more type I and 2 times more type VI per unit area) both on the leaves and the main vegetative stem (Fig. 5A, B).

A



B

Trichome type	Controll	idm3 CRISPR line (guide1)	idm3 CRISPR line (guide2)	Fold change
I	2	4,133	6,13	2-3x
VI	16,06	30,26	29,73	2x

Figure 5: A. Cross-section view of tomato stem made by SEM (red circle- trichome type I; green circle- trichome type VI.
B. idm3 CRISPR line has increased number of type I (long) and type VI (small glandular) trichomes per unit area.

Indeed we could also detect morphological deffects of type I trichome, with abnormal branching in the adaxial part of the leaves of idm3 CRISPR lines (Fig.6 A, B). These phenotypic marks of idm3 lines suggest that IDM3 act as a negative regulator of glandular trichome formation and it might have role not only in trichome development but also in the regulation of trichome morphology.

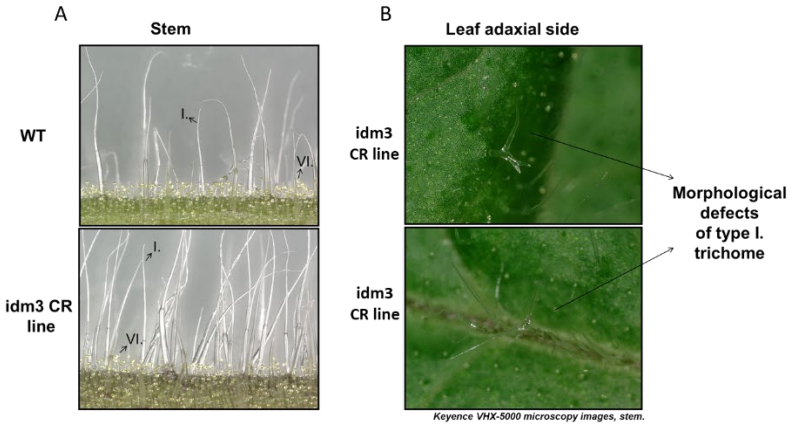


Figure 6: A. The density of type I and type VI glandular trichomes are increased on the stem of idm3 CRISPR lines.
B. On the adaxial side of the leaves of idm3 CRISPR lines morphological defects of type I trichomes occours.
Keyence VHX-5000 microscopy images.

2.2 **idm3 CRISPR lines accumulate acyl sugars**

Type VI glandular trichomes are abundant on the adaxial surfaces of the leaves and stems of idm3 CRISPR lines. Type-VI glandular trichomes produce and secrete terpenoids, that occupy a major role in tomato defenses, as they can be directly toxic or repellent to insect pests (6). Notably, artificial induction of type-VI glandular trichomes and their associated terpene volatiles increase the level of resistance against diverse herbivorous arthropods (20). We performed gas chromatography-mass spectrometry (GC-MS) measurements to analyse whether the increased level of type VI trichome in idm3 CRISPR lines is associated with terpenoid accumulation. Leaves taken from the sixth internode (counted from the shoot tip) and stem chopped from the sixth internode of 6 weeks old tomato plants were dipped in 3 ml of hexane for 5 min to extract trichome exudates. Hexane-extracted compounds with tetradecane internal standard were quantified by GC-MS (HP Agilent 6890 GC and 5975 MS, Agilent

Technologies) according to the procedure described by Zehao Gong et al. (21). In total 12 terpenoids were detected in the leaf extracts and the concentration of one monoterpene (cis-B-ocimene) and two sesquiterpene (gammana-elemene, trans-B-caryophyllenne) were slightly increased in idm3 CRISPR lines compared to the WT. The level of seven terpenoids detected from stem samples were not changed (Fig 7). Based on these measurements we conclude that moderate increase of type VI glandular trichomes does not result in terpenoid accumulation. We conclude that similar to hairless mutant (22), idm3 CRISPR line might be also characterised by deficient accumulation of terpenes in type-VI trichomes. This might be due to the disturbed expression of terpene synthase genes (see later in part 2.3).

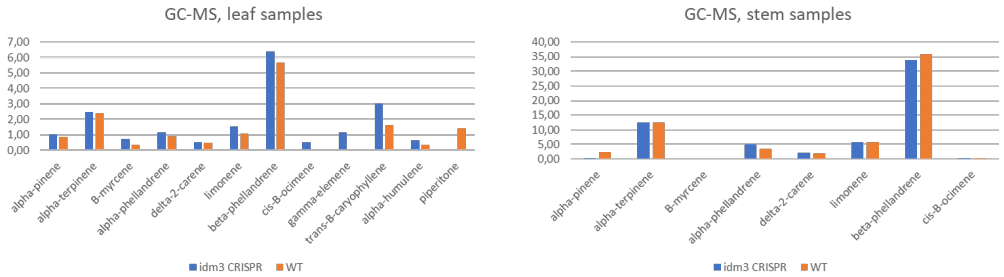


Figure 7: Results of GC-MS measurements of terpenoids from six weeks old WT and idm3 CRISPR lines. Leaf samples were normalised to the measured gramm/sample, while in case of stem samples we normalised to the volume of cylinder. Note that samples for GC-MS were taken from one WT and one idm3 CRISPR line thus significance of the differences are still not reliable (new measurements with 5 biological replicates are in progress).

Acyl sugars (AS) are abundant trichome specific metabolites synthetized and stored in type I and IV glandular trichomes and they can deter or repel aphids, leaf miners, they are excellent emulsifiers that adhere to arthropod cuticles to immobilize them (7). Since the density of type I trichomes are 3-4 times higher on idm3 CRIPSR lines, we suppose that the amount of the produced metabolites would correlate to the density of glandular trichomes. We used high-performance liquid chromatography (HPLC) to measure whether the acyl sugar content of idm3 mutants are increased. Ten leaflets (from the node adjacent to the apical tissue of six weeks old plants) or 8 stem pieces (6th internode of six weeks old plants) per sample were harvested for UHPLC/MS analyses. UHPLC/MS were performed using a single quadrupole electrospray ionization mass spectrometer (LCMS-2020, Shimadzu). Notably the level of previously identified tetraacylsucroses (based on their retention time: 666, 680, 709, 722, 736, 750, 751, 764, 778); 23) were higher in idm3 mutant leaves while only three tetraacylsucroses (retention time: 666, 680, 750) were increased in stem samples (Fig.8).

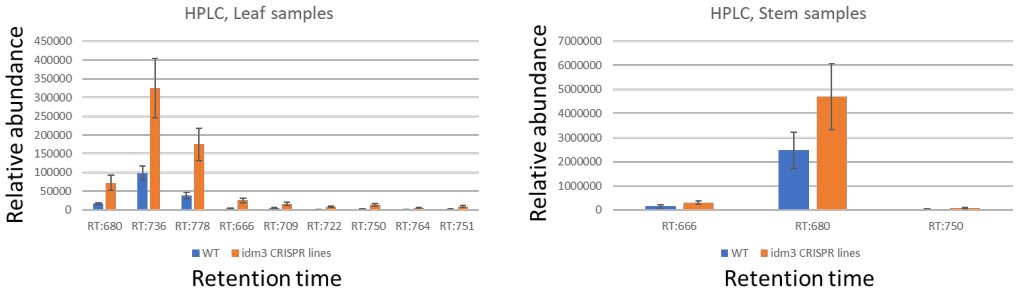


Figure 8: Results of HPLC measurements of acyl sugars from six weeks old WT and idm3 CRISPR lines. Leaf samples were normalised to the measured gramm/ sample, while in case of stems samples we normalised to the volume of cylinder. Mean values were calculated from five independent samples (n = 5) and the acyl sugar level of the mutants are shown relative to the WT line.

These results suggest that the increased type I trichome density on idm3 CRISPR lines results in elevated acyl sugar content compared to the wild-type tomato plants. Current understanding of how acyl sugar yield differences in different plant tissues (for examples in leaf and stem) is still not known (23). Further experiments would be necessary to directly collect metabolites from type I trichome gland and reliably identifiy by ultra-high performance liquid chromatography–mass spectrometry combined NMR spectroscopy (UHPLC/MS-NMR).

2.3 JAZ2 has role in idm3-dependent trichome formation

Glandular trichome initiation is known to be regulated by plant hormones (24). In response to pathogen stress jazmonate signalling (JA) induce glandular trichome initiation. In tomato the JA signalling repressor SlJAZ2 (Solyc12g009220) inhibit several transcription factors involved in glandular trichome initiation. Transgenic overexpression (OE) of SlJAZ2 in tomato strongly decreases glandular trichome density (especially trichome VI is decreased in SlJAZ2 OE lines) (24). Our scanning electron microscopic comparison of tomato leaves and stems show that relative to wild-type, idm3 CRISPR lines display a significant increase in the density of I, VI glandular trichome types (2-4 fold increase). We have analysed the methylation status of JAZ2 promoter and intron region and we have shown that both regions were hypermethylated in idm3 lines (Fig. 9A). Accordingly, JAZ2 mRNA level was decreased in idm3 lines suggesting that the increased trichome number of idm3 CRISPR lines might be related to perturbation of JAZ2 expression (Fig.9B).

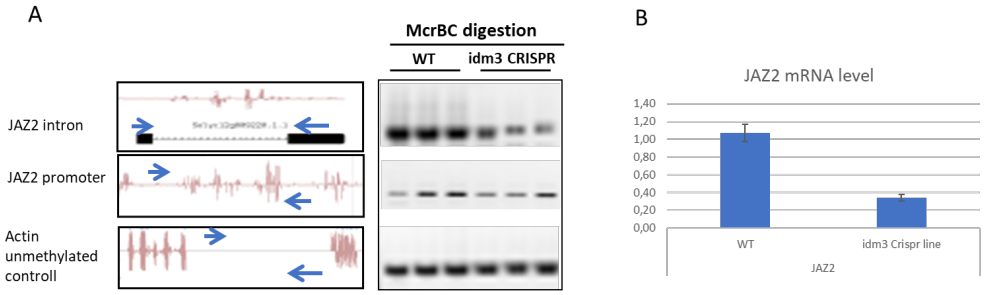


Figure 9: A. Methylation sensitive CHOP-PCR. Genomic DNA were isolated form WT and idm3 CRIPSR line stems (from 6th internode of six weeks old plants). After McrBC (methylation sensitive enzyme, cuts only if cytozines are methylated) digestion, DNA were used for PCR with the flanking primer pairs (shown by blue arrows). PCR amplification is less efficient from hypermethylated regions. Unmethylated region of ACTIN gene were used as negative controll. B. qPCR measurement of JAZ2 mRNA level from WT and idm3 CRISPR lines. Mean values were calculated from five independent samples (n = 5).

These findings identify tomato IDM3 as a novel epigenetic regulator of glandular trichome formation. Notably, similar to idm3-, idm1 CRISPR lines (IDM1- Solyc07g062600) also show higher glandular trichome density suggesting that the SlIDM complex might have role in trichome formation.

2.4 Gene expression changes promoted by IDM3 mutation

With the aim to determine the expression pattern of IDM3, we perfomed qRT-PCR using total RNA extracted from several tissues of WT plants, which included 2 weeks old seedlings; juvenile (4 weeks old) root, stem and leaf; mature root, stem, leaf; flower and fruits at different maturation stages. Expression patterns confirmed that IDM3 is constitutively expressed in all analysed tissues, reaching the highest expression level during fruit ripening (Fig. 10A). Since we could not detect any developmental/ ripening phenotype of fruits on idm3 CRISPR lines we suppose that IDM3 might have redundant role in fruit development. We could detect two other IDM3-like gene in

tomato genome however we still not analysed their expression pattern. The expression of the genes known to be involved in tomato trichome development, i.e. Woolly, SlCycB2, SlMixta-like, SlMYC1 was also analysed in vegetative stems of WT and idm3 mutant plants with the aim to explore possible interactions with IDM3. However, no significant differences in relative expression were found among these genes (data not shown) suggesting that IDM3 might control glandular trichome density by a signal transmission pathway in which the analysed genes seem not to be involved. Thus, the molecular background how IDM3 participate in trichome formation was further investigated using RNA sequencing of idm3 CRISPR mutant and WT vegetative stems (3 replicates of WT were compared to the 3 replicates of idm3 mutant transcriptome). As expected (see 2.1 part), IDM3 mRNA level was significantly decreased in idm3 CRISPR line compared to the WT (Fig. 10 C). According to the qPCR data, the level of known trichome regulators (see above) were not changed. Overall, this analysis allowed to identify 273 downregulated and 181 upregulated genes in mutant plants that showed at least a twofold change in transcript number (Fig. 10B). Among the upregulated genes, were found several glycosyltransferases (Solyc08g006350.3, Solyc08g006410.5, Solyc08g006330.3) that beside their important role in anthocyanin synthesis (25), catalyze glycosylation of plant secondary metabolites like acyl sugars (26). This is in accordance with our results that acylsugar content of idm3 lines is higher compared to the WT (see 2.2 part). Another interesting upregulated gene in idm3 CRISPR lines is NRPD2B (Solyc08g075940.4) encoding the major subunits of Pol IV and Pol V polymerases involved in RNA directed DNA methylation pathway (RdDM) (27) through the generation of 24-nt small RNAs (see further interpretation in „Plans for this year" part) (Fig.10B). Disease resistance protein (CC-NBS-LRR class) family protein (Solyc12g044190) were also significantly upregulated suggesting that idm3 CRISPR line might be more resistant to different abiotic stresses (Fig.10C). Among the downregulated genes were found JAZ2 as expected, however it shows lower decrease in idm3 lines compared to our previous qPCR measurements (Fig.10C). The level of other JAZ genes (13 JAZ gene were described in tomato) were not changed. In addition Solyc08g005640.4 were also found to be repressed in idm3 mutants. Solyc08g005640.4 is a homologue of the Arabidopsis TERPENE SYNTHASE 21 (AT5G23960), encoding a sesquiterpene synthase (28) which support our GC-MS results that despite of the increase of type VI trichome density, the level of terpene metabolites are only slightly higher in idm3 mutants (Fig 10C). Another interesting downregulated gene is the filament-like plant protein 4 (Solyc07g054970) (it is a microtubule-binding protein that regulate trichome cell shape (TCS1) in Arabidopsis) wich might interpret the morphological deffects of type I trichomes we detected on the leaves of idm3 CRISPR lines. Downregulation of two genes, the regulator of chromosome condensation (RCC1) family protein (Solyc03g005590) and DNA-3-methyladenine glycosylase support our hypothesis that IDM3 affect the density of glandular trichomes through an epigenetic regulation.

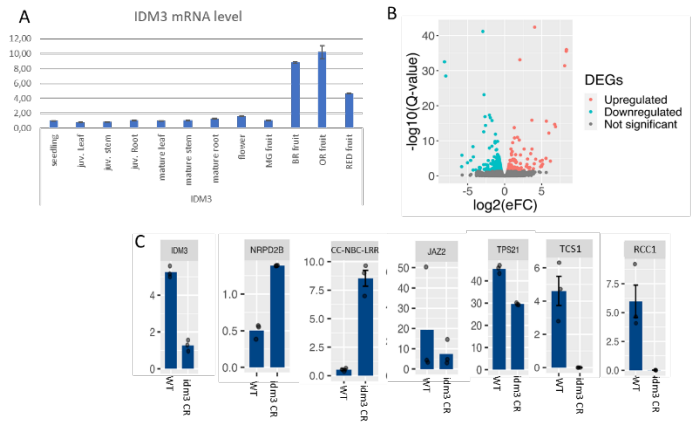


Figure 10: A. Tissue specific expression of IDM3 in tomato. Relative expression was determined by qRT-PCR using three biological replicates of different tissues from WT tomato plants. B. The level 273 downregulated and 181 upregulated genes in mutant plants that showed at least a twofold change in transcript number. C. RNA sequencing analysis performed from WT and idm3 CRISPR lines demonstrates that IDM3 is repressed in idm3 CRISPR mutants. NRPD2B (Solyc08g075940), JAZ2 (Solyc12g009220), TPS21 (Solyc08g005640), TCS1(Solyc07g054970), CC-NBC-LRR (Solyc12g044190), RCC1 (Solyc03g005590) showed significant differences in their relative expression in idm3 CRISPR lines. Three biological replicates of stem tissues were collected for RNA sequencing analysis.

2.5 IDM3 induces epigenetic changes in the tomato genome

Arabidopsis proteins homologue to IDM3 (Increased DNA Methylation 3) is a chaperon component of IDM complex that seem to have role in targeting of ROS1 DNA demethylase to prevent DNA hypermethylation (1). Arabidopsis idm3 T-DNA mutants show DNA hypermethylation, particularly at transposable elements (TEs) and other repeats (1). Thus we decided to analyse the epigenetic modifications caused by idm3 mutant lines through the whole-genome bisulfite sequencing of genomic DNA (WGBS) from the same vegetative stem of idm3 and WT plants analysed by RNA-seq (3 replicates of WT were compared to the 3 replicates of idm3 mutant DNA). A total amount of 9501 differentialy methylated cytosines (DMCs) were obtained from wich hypomethylated regions were dominant in idm3 mutant plants (Fig.11A).

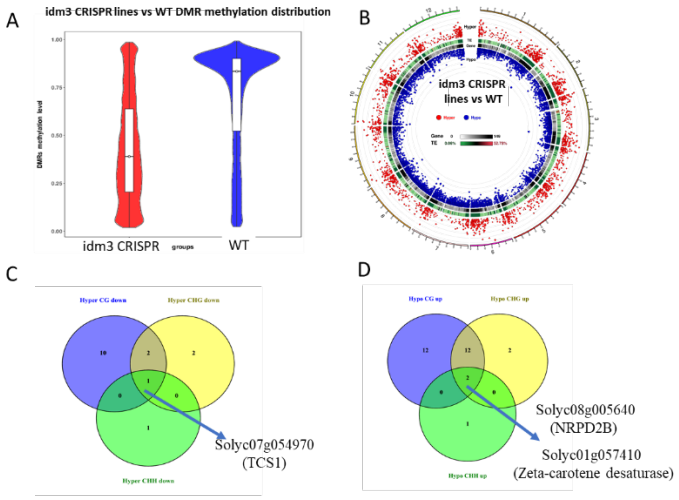


Figure 11: A. Violin plot for DMR methylation level in three contexts (CG, CHG, CHH). IDM3 CRISPR line is hypomethylated compared to the WT. B Circos plot for DMR condition in three contexts (CG, CHG, CHH). Hypermethylation is shown by red point, hypomethylation are shown by blue points. The heatmap shows gene density. C. Hypermethylated downregulated genes. D. Hypomethylated upregulated genes. Three biological replicates of stem tissues were collected for WGBS sequencing analysis.

The DMCs identified in the genome of idm3 mutants were mainly located in the euchromatic telomeric portion of the 12 tomato chromosomes mostly within promoter, exon and intron regions (Fig. 11B). We could detect only a

few statistically significant correlation between the position of the DMCs and the DEGs. Solyc01g057410 (zeta-carotene desaturase) and Solyc08g075940.4 (NRPD2B) genes were upregulated in idm3 mutants and they holds a DMC in its promoter region which is demethylated in all sequence context (CG,CHG,CHH) in idm3 and hypermethylated in WT plants (Fig. 11D). Only one down-regulated genes were found in idm3 mutant plants: TCS1 (Solyc07g054970) has DMCs in its promoter hypermethylated in all sequence context (CG,CHG,CHH) in idm3 lines compared to the WT (Fig.11C). Overall, idm3 mutation affect DNA methylation but in contrast to Arabidopsis idm3 mutants, in our idm3 CRISPR lines hypomethylation is more prominent thus tomato IDM3 might be a negative regulator of DNA demethylation (affecting all 3 context). We speculate that IDM3 might have role not only in DNA demethylation but as a small chaperon protein it might be also a component of DNA methylation complex. This hypothesis is supported by the RNA seq results that the main component of RdDM pathyway, NRPD2B, is upregulated in idm3 CRISPR lines. Indeed we have generated dml2 CRISPR lines in our lab but we could not detect any difference in trichome density compared to the WT. Remarkably, idm1 CRISPR lines also show higher glandular trichome density. These results further support the idea that IDM3 regulate trichome density independently from DML2 DNA demethylation, perhaps as a component of IDM complex. TCS1 and NRPD2B are two good candidate to further investigate IDM3 role in trichome development.

2.6 IDM3 has role in heat shock response in tomato

Similar to its *Arabidopsis* orthologue, tomato IDM3 is also a small peptide with a HSP20 heat shock domain and it belongs to the familiy of small heat shock proteins (Fig.12A). In addition, we could identify two classical heat response element (HRE) in tomato IDM3 promoter region. Although IDM3 in Arabidopsis is not heat inducible (1) we have analysed whether IDM3 has any role in heat response. We have made heat shock experiments of 10 days old WT tomato seedlings at 25°C, 40°C and 42°C and collect RNA samples after 1 hour, 4 hour and 24 hour of heat treatement. qPCR measurements reveal that IDM3 was highly induced at 40° and 42°C after 1, 4, 24 hour (Fig.12B). Indeed IDM3 mRNA level was slightly increased in plants that were grown for longer time at ambient elevated temperature (30°C) suggesting that IDM3 might have role in heat stress response. We are now testing the heat tolerance of idm3 mutants expecting that if IDM3 has role in heat response than idm3 CRIPSR lines might be less tolerant to heat treatment compared to the WT.

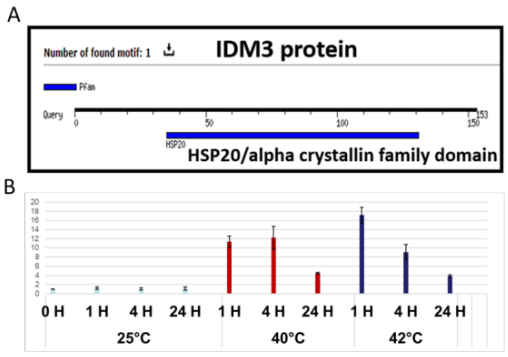


Figure 12: A. Schematic, representation of the domain structure of tomato IDM3 protein. B qPCR measurement of IDM3 mRNA level from heat shocked WT tomato plants. Mean values were calculated from five independent samples (n = 5).

Plans for this year (2022)

1) Protein interaction studies: We will make transient agroinfiltration based co-immunoprecipitation assay to demonstrate the interaction between SLIDM1 and SlIDM3 (Myc-, HA and GFP TAG-ed versions of SlIDM1- and SlIDM3). We had problem with cloning and transiently expressing the 3,5 kb long IDM1 gene, but constructs were finally prepared and IP experiments are in progress. Indeed, we have already generated 35S-GFP and 35S-IDM3-GFP transgenic tomato plants and use them to perform protein mass spectrometry to identify in vivo protein interactions. 35S-IDM3-GFP overexpressing plants are also used to measure and compare the level of genes that were changed in RNA seq results of idm3 mutant CRISPR lines.

2) Complementation and localisation studies: 35S-IDM3-GFP fusion construct were also used to complement our idm3 CRISPR lines. In 2022 summer, we will phenotype the complemented T0 lines and we will also examine the subcellular localization of the IDM3 protein.

3) Pest damage tests: terpenoids and acyl sugar synthesised and stored in type VI and type I glandular trichomes respectively, are critical against pathogen attacks. Accordingly, the increased density of these trichomes was shown to enhance tolerance to spider mites (*Tetranychus urticae*) and potato aphids (*Macrosiphum euphorbiae*) (29, 30, 31). In collaboration with the Center for Agricultural Research (ATK-Növényvédelmi intézet) institute we make spider mites infection tests to reveal whether the higher trichome density of idm3 CRISPR lines confer tolerance to pests (experiment planned to finish during 2022 summer). This is an important but time-consuming experiment, depending also on weather conditions, since pests are more active and viable during the summer even under laboratory conditions. Indeed, our greenhouse conditions are ideal for tomato growth only from May to September. Since the trichome density strongly depends on the temperature and light conditions we decided to wait and perform this experiment during this summer.

4) Bioinformatic analysis of high-throughput sequencing data: both the RNA seq and WGBS results were completed in May 2022 thus we still have to achieve a detailed analysis of data in the next few months and validate the results using qPCR and CHOP-PCR validation techniques.

5) NRPD2B (Solyc08g075940.4), an important component of RdDM pathway (27) is upregulated in idm3 CRISPR mutants. In line with another research project, we have already generated nrpd2b CRISPR mutants in our lab thus we will use this line to reveal how idm3 can influence the expression of NRPD2B (we will compare the phenotype of nrpd2b- and idm3 mutant plants and perform qPCR measurements and sRNA northern blots).

6) Testing the heat tolerance of idm3 CRISPR line are in progress

7) At the end of 2022 we plan to send the manuscript for publication into “Horticulture Research” journal (IF: 6,072).

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