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Study of RNAi Mechanisms in Tobacco BY-2 Cell Line and Potato Plants

Studium mechanizmů RNAi v tabákové buněčné linii BY-2 a rostlinách lilku bramboru

Doctoral thesis

Supervisor: RNDr. Lukáš Fischer, Ph.D.

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DECLARATIONS

I hereby declare that this Ph.D. thesis documents my own work and I wrote it independently. This work or any substantial part of the text is not a subject of any other defending procedure. I declare that all used sources were cited and acknowledged properly.

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ABSTRACT

Knowledge of the processes of RNA interference, the regulation of gene expression by small RNAs (sRNAs), has grown at an unprecedented rate over the last 30 years. Some of the findings were literally revolutionary, as they revealed events that overturned many long-held notions. Many phenomena have been shown to be highly conserved and common to organisms of different species, but others are specific to certain lineages or have not yet been fully explored. There is also a lack of knowledge about the interconnection of numerous pathways – for example between silencing at the transcriptional (TGS, leading to the promoter methylation) and post-transcriptional levels (PTGS, affecting mRNA stability or translation). The present work summarizes the findings of two published and two unpublished works and attempts to describe some of the less known sites of RNA interference using various plant model organisms.

Research on *Solanum tuberosum* transgenic lines has revealed the ability of 5-azacytidine to restore the expression of transcriptionally silenced transgenes at the whole plant level. *De novo* regeneration from leaves of such plants can lead to re-silencing of reactivated transgenes and thus serves as a selection method to exclude lines prone to spontaneous silencing. The nature of changes in the expression of the two reporter genes indicated the coupling of PTGS and TGS, but also, the possibility of a gradual spread of methylation along the inserted T-DNA. Therefore, further research was aimed for the induction of PTGS and TGS at the cellular level in *Nicotiana tabaccum* BY-2 lines. Both approaches led to the generation of specific sRNAs matching predominantly to the target locus region, but sRNAs of a transitive nature outside the target locus also emerged. In particular, sRNAs from the terminator region could thus play a role in the propagation of methylation along the T-DNA, since the same terminator was used multiple times. The methylation of target loci was otherwise very accurate and did not spread to its surroundings during the monitored 10-14 days.

In the last part of the work, I focused on proteins SAG18 and aPHC from *Arabidopsis thaliana* with a certain homology to the animal transmembrane dsRNA transporter, the protein SID-1. The study of SAG18 function in BY-2 cells did not demonstrate the effect of externally added sRNAs on the level of transcription of the targeted transgene, but the same negative results were obtained with SID-1 transporter from *Caenorhabditis elegans*. Analyses of double mutant plants in *SAG18* and *aPHC* showed no significant changes in phenotype, but only indicated their possible role in the function of stomata guard cells.

Key words: RNA interference, gene silencing, PTGS, TGS, sRNAs, transmembrane sRNAs transport, 5-azacytidine

ABSTRAKT

Znalosti o procesech RNA interference, tedy regulace genové exprese prostřednictvím malých RNA (sRNA), se za posledních 30 let nebývale rozrostly. Některá zjištění byla doslova revoluční, neboť odhalila děje, které převrátily mnohé dosud zažité představy. Řada jevů se ukázala být značně konzervovaná a společná různým druhům organismů, jiné jsou však specifické pro určité vývojové větve či dosud ne zcela prozkoumané. Chybí také znalost o propojení četných drah – kupříkladu mezi umlčováním na transkripční (TGS, vedoucí k metylaci promotoru) a posttranskripční úrovni (PTGS, ovlivňující stabilitu mRNA či translaci). Předkládaná práce shrnuje poznatky dvou publikovaných a dvou dosud nepublikovaných prací a pokouší se prostřednictvím různých rostlinných modelových organismů popsat některá z méně známých míst RNA interference.

Výzkum na transgenních liniích *Solanum tuberosum* odhalil možnost obnovit pomocí 5-azacytidinu expresi transkripčně umlčených transgenů na úrovni celých rostlin. Regenerace *de novo* z listů takovýchto rostlin může vést k opětovnému umlčení reaktivovaných transgenů a sloužit tak jako selekční metoda pro vyřazení linií náchylných k samovolnému umlčení. Charakter změn v expresi dvou sledovaných reportérových genů naznačoval spřažení PTGS a TGS, ale také, možnost postupného šíření metylace v rámci vnesené T-DNA. Další výzkum se proto věnoval na buněčné úrovni cílené indukci PTGS a TGS v liniích *Nicotiana tabaccum* BY-2. Oba přístupy vedly k tvorbě specifických sRNA cílených převážně do oblasti cílového lokusu, avšak objevily se též sRNA transitivní povahy, mimo cílový lokus. Zejména sRNA z oblasti terminátoru tak mohly hrát roli právě v šíření metylace podél T-DNA, neboť stejný terminátor byl použit vícekrát. Metylace cílového místa byla jinak velmi precizně cílená a nešířila se během sledovaných 10-14 dnů do svého okolí.

V poslední části práce jsem se zaměřil na proteiny SAG18 a aPHC z *Arabidopsis thaliana* s určitou homologií k živočišnému transmembránovému přenašeči dsRNA, proteinu SID-1. Studium funkce SAG18 v buňkách BY-2 nevedlo k prokázání vlivu externě přidaných sRNA na úroveň transkripce cíleného transgenu, stejné negativní výsledky byly získány i při testování SID-1 transportéru z *Caenorhabditis elegans*. Analýzy dvojitě mutantních rostlin v *SAG18* a *aPHC* také nevykazovaly žádné výrazné změny ve fenotypu, byla pouze naznačena jejich možná role ve fungování svěracích buněk průduchů.

Klíčová slova: RNA interference, umlčování genů, PTGS, TGS, sRNA, transmembránový transport sRNA, 5-azacytidin

ABBREVIATIONS

AA	amino acid
AGO1-10	argonaute protein 1-10
aPHC	putative alkaline phytoceramidase
AS	antisense
AzaC	5-Aza-2´deoxycytidin
BS	bisulphite
C. elegans	*Caenorhabditis elegans*
CaMV 35S, P35S	Cauliflower mosaic virus 35S constitutive promoter
CMT2, 3	chromomethylase 2, 3
DCL1, 2, 3, 4	dicer-like protein 1, 2, 3, 4
DDM1	decrease in DNA methylation 1
DME	demeter
DML2, 3	demeter-like 2, 3
DNMT1	DNA methyltransferase 1
DPI	day post inoculation
DRD1	defective in RNA-directed DNA methylation 1
DRM1, 2	domains rearranged methyltransferase 1, 2
dsRNA	double-stranded RNA
FP	fluorescent proteins
GFP	green fluorescent protein
HEN1	HUA enhancer 1
HIGS	host-induced gene silencing
hc-siRNA	heterochromatic siRNA
hpt	hygromycin phosphotransferase
H3K9me	methylation of lysine 9 in histone 3
IR	inverted repeat
KAN	kanamycine
LD	long day
LMW	low molecular weight
mC	methylcytosine
mRNA	messenger RNA

MIR	microRNA gene
miRNA	small microRNA
MET1	methyltransferase 1
nat-siRNA	natural *cis*-antisense transcript siRNA
nptII	neomycin phosphotransferase
ORF	open reading frame
PD	plasmodesmata
Pol II, IV, V	DNA-dependent RNA polymerase II, IV, V
PPFD	photosynthetic photon flux density
pri-miRNA	primary small microRNA
PTGS	posttranscriptional gene silencing
RdDM	small RNA-directed DNA methylation
RDR1, 2, 6	RNA-dependent RNA polymerase 1, 2, 6
RNAi	RNA interference
ROS1	repressor of silencing 1
RT-qPCR	quantitative real-time PCR
SAG18	senescence associated gene 18
SD	standard deviation
SID	systemic RNAi defective
sRNA	small ribonucleic acid
sRNA*	non-guide passenger strand of small RNA
SGS3	suppressor of gene silencing 3
TAS	*trans*-acting non-coding genomic loci
ta-siRNA	*trans*-acting siRNA
TE	transposable element
TGS	transcriptional gene silencing
UT	unterminated
v-siRNA	virus derived siRNA
VIGS	virus-induced gene silencing

CONTENT OF THE THESIS

1. INTRODUCTION

Plants have always been of great importance to mankind. Directly as a source of food, energy, building material, clothes or indirectly as a main producer of organic compound in the Earth's ecosystem, an oxygen producer, a habitat creator or even an art inspirator. Their deeper study can also reveal some basic mechanisms, crucial for better understanding not only plant internal processes like photosynthesis, the key process for converting of light energy into chemical energy, but also core molecular apparatus shared by various eukaryotic cells. Although a lot of crucial studies is conducted on animal cells and then applied in a plant system, sometimes this order is inversed.

Indeed, in 1990 Carolyn Napoli and her colleagues described RNA interference (RNAi) phenomenon by trying to overexpress chalcone synthase in pigmented petunia petals. Surprisingly, almost a half of plants showed completely unexpected phenotype – white flowering pattern (Napoli et al., 1990). Nevertheless antisense RNA was used for a gene downregulation much earlier (Izant and Weintraub, 1984). These "small steps" together have created a scaffold for detailed elemental RNAi characterization few years later by the team of Andrew Fire in research with microinjection of RNA into nematode *Caenorhabditis elegans* (Fire et al., 1998). The very same year few months later have Waterhouse et al. showed the same results also in plants. Finally, in 2006 Andrew Fire together with Craig C. Mello won the Nobel Prize in Physiology or Medicine for uncovering the process of RNA interference.

Nowadays we know that the main purpose of this biochemical mechanism is to protect genome against invasive viral (Ratcliff et al., 1997), transposon (TE; Henderson and Jacobsen, 2007) or transgene (Wassenegger et al., 1994) sequences and to regulate gene expression during plant development (Boerjan et al., 1994) and response to various stresses (Navarro et al., 2006). In the course of time the knowledge about RNAi exponentially grows and many applied biotechnological methods use its background. Nevertheless, several fundamental questions regarding RNAi processes remain unanswered, therefore this thesis is trying to shed light on some of them.

1.1. The general principles of plant RNAi

Plant RNAi is ingenious and very diverse set of pathways taking into consideration the existence of several plant protein paralogs and different regulatory routes. Nevertheless, basic machinery remains evolutionary conserved for most eukaryotic organisms – the regulation of gene expression by short non-coding 20-24 nt RNAs (sRNAs; Baulcombe, 2004).

sRNAs can be divided into two basic groups – microRNAs (miRNAs; Rhoades et al., 2002) and small interfering RNAs (siRNAs; Elbashir et al., 2001; Hamilton and Baulcombe, 1999; Ye et al., 2012). These crucial informative molecules are produced in cells by different routes, mostly from internal or external double stranded RNA (dsRNA) precursor by the enzymatic activity of an endoribonuclease of DICER-like (DCL) families (Schauer et al., 2002). Short sRNA duplexes gain 2nt 3´overhangs folowed by 2´*O*-methylation managed by the activity of enzyme HUA enhancer 1 (HEN1; Fig. 1.4.2a; Li et al., 2005). Guide strand of sRNA is then loaded into ARGONAUTE (AGO) protein creating effector ribonucleoprotein complex called RISC (RNA-induced silencing complex). Subsequently the expression of homologous sequences can be negatively affected by sRNA Watson-Crick base paring recognition in posttranscriptional gene silencing (PTGS; by mRNA cleavage or inhibition of protein translation; Baumberger and Baulcombe, 2005; Brodersen et al., 2008) or transcriptional gene silencing (TGS; by DNA or histone modification; Sijen et al., 2001; Vaucheret and Fagard, 2001). Some pathways use RNA-dependent RNA polymerases (RDRs) creating or even amplifying the sRNA silencing signal (Voinnet, 2008).

1.2. sRNA families and its biogenesis

We can differentiate three major routes of sRNA biogenesis in flowering plants – 21-22 nt siRNAs produced by DCL2 and DCL4, 20-22 nt miRNAs made mainly by DCL1 and heterochromatic siRNAs (hc-siRNAs) formed by DCL3 activity. All these routes share unifying biochemical steps: i) dsRNA precursor formation, ii) dsRNA processing by DCL proteins, iii) non-guide passenger strand (sRNA*) removal and RISC effector complex formation, iv) complementary RNA/DNA recognition and its subsequent regulation (see Fig. 1.2; reviewed in Lee and Carroll, 2018).

One of the main differences in sRNAs is the origin of their dsRNA precursor. For example, **miRNAs** (Fig. 1.2) are produced from primary single-stranded microRNA (pri-miRNA) intermediates during complex enzymatic pathway in Cajal bodies (Fujioka et al., 2007). Pri-miRNA with its complicated imperfect secondary hairpin structure arises by the activity of polymerase II (Pol II) from non-coding microRNA genes (*MIR*; Reinhart et al., 2002; Lee et al., 2004). Each gene gives only one miRNA (Meyers et al., 2008). The most essential for their biogenesis is DCL1 protein, but also DCL4 for generating an evolutionarily recent miRNAs in *Arabidopsis thaliana* (Amor et al., 2009). Matured miRNAs associate with AGO1 and are exported to the cytoplasm (Bologna et al., 2009, 2018) to guide usually PTGS during developmental processes through mRNA cleavage or translational repression (Rhoades et al., 2002; Bartel et al., 2004). Relatively rare subgroup of *A. thaliana*, *Oryza sativa* and *Physcomitrella patens* longer miRNAs (23-27 nt) is also processed by DCL3 in collaboration with

RDR2 and Pol IV and associate with AGO4 controlling TGS. Nonetheless their origin is derived from *MIR* genes in contrast to hc-siRNA that are processed in a different way (see below; Vazquez et al., 2008; Chellappan et al., 2010).

miRNAs are also important for production of 21 nt *trans*-acting siRNAs (see Fig. 1.2). These **ta-siRNA** are derived from transcripts of endogenous non-coding *TAS* genes (Allen et al., 2005). Cleavage of miRNA and RDR6, DCL2/4/5 and AGO1/7 involvement are essential for their biogenesis (Peragine, 2004; Yoshikawa et al., 2005). In this process called transitivity, an initial pool of primary sRNAs (for example DCL1-dependent miRNA), directed against one region of a transcript, induces production of secondary siRNAs matching to dsRNA sector outside the primary RNA target sites (Yoshikawa et al., 2005). This remarkable system is highly dependent on suppressor of gene silencing 3 (SGS3; Yoshikawa et al., 2013) and is involved in the regulation of plant development (summarized in Singh et al., 2018).

Generally, siRNAs are derived from long dsRNA precursors with perfect or near-perfect complementarity and diverse genesis. The most abundant class of siRNA are **hc-siRNA,** derived from heterochromatic regions and associated with deposition of repressive chromatin modifications at target DNA loci (see more in Chapter 1.4.2. and in Fig. 1.2. and 1.4.2a). Since their length is mostly 23-24 nt, they can be easily distinguished from other classes of endogenous plant small RNAs (Xie et al., 2004). hc-siRNAs have very strict requirements for specific protein members of the RDR, DCL and AGO families – for biogenesis Pol IV (Onodera et al., 2005; Wierzbicki et al., 2009; Haag and Pikaard, 2011) with subsequent processing by RDR2 and DCL3 (Xie et al., 2004) and for function (target recognition) AGO3, 4, 6 or 9 (Qi et al., 2006; Havecker et al., 2010).

The third most common group of siRNAs with endogenous origin are **nat-siRNAs** (Fig. 1.2). Compared to other types of siRNAs, which need RDR enzymes for dsRNA precursor synthesis, dsRNA precursors of 21-22 nt nat-siRNAs are thought to originate from the hybridization of independently transcribed complementary RNAs. Depending on their formation from transcripts off overlapping or non-overlapping genes we can differentiate *cis*- (transcripts from the same locus) or *trans*-nat-siRNAs. They often play an important role in the plant response to biotic or abiotic stresses (Borsani et al., 2005; Katiyar-Agarwal et al., 2007; Zhou et al., 2009). RDR-independent mechanism of endogenous siRNA formation is also used in case of naturally inverted genes, that are curiously abundant in *A. thaliana* genome and are thought to be an evolutionary precursors for *MIR* genes (Wang et al., 2011).

RNA silencing can be also initiated by dsRNA of external provenance – derived from viruses or transgenes (see Chapter 1.5. and Fig.1.2). Virus derived siRNA (**v-siRNA**) formation can by triggered in a host by the presence of both RNA and DNA viruses (Pyott and Molnar,

2015). dsRNA processing is particularly driven by DCL2, DCL4 (sometimes also DCL1 and 3; Blevins et al., 2006; Deleris et al., 2006) and RDR1/6 (Wang et al., 2010). Emerging 21, 22, 24 nt v-siRNAs associate with AGO1/2/7 and mediate PTGS or TGS of virus nucleic acids (Burgyán and Havelda, 2011). Deeper knowledge of RNA silencing mechanisms has enabled to evolve powerful tools for virus-resistant crop engineering during last decades (Abel et al., 1986; Scorza, 1994; Shekhawat et al., 2012), even though diverse plant viruses have specific "counter-defensive" proteins that suppress RNAi apparatus and enable viral replication and plant infection (Voinnet, 2005a; Lakatos et al., 2006; Guo et al., 2019).

Transgene-derived siRNAs could by spontaneously created from aberrant *sense* transcripts by the activity of RDR polymerase (Fig. 1.2; Baulcombe, 2004). Aberrant can mean missing 5´cap, incorrect termination or even wrong folding (Gazzani et al., 2004; Herr et al., 2006; Luo and Chen, 2007). siRNAs have typically 20-22 nt in length (produced by DCL2, 4 and associated with AGO1 in transgene mRNA cleavage) and 24 nt (AGO4 and RDR2 mediated small RNA-directed DNA methylation, RdDM; reed more in Chapter 1.4.2.; Mlotshwa et al., 2008; Wroblewski et al., 2014).

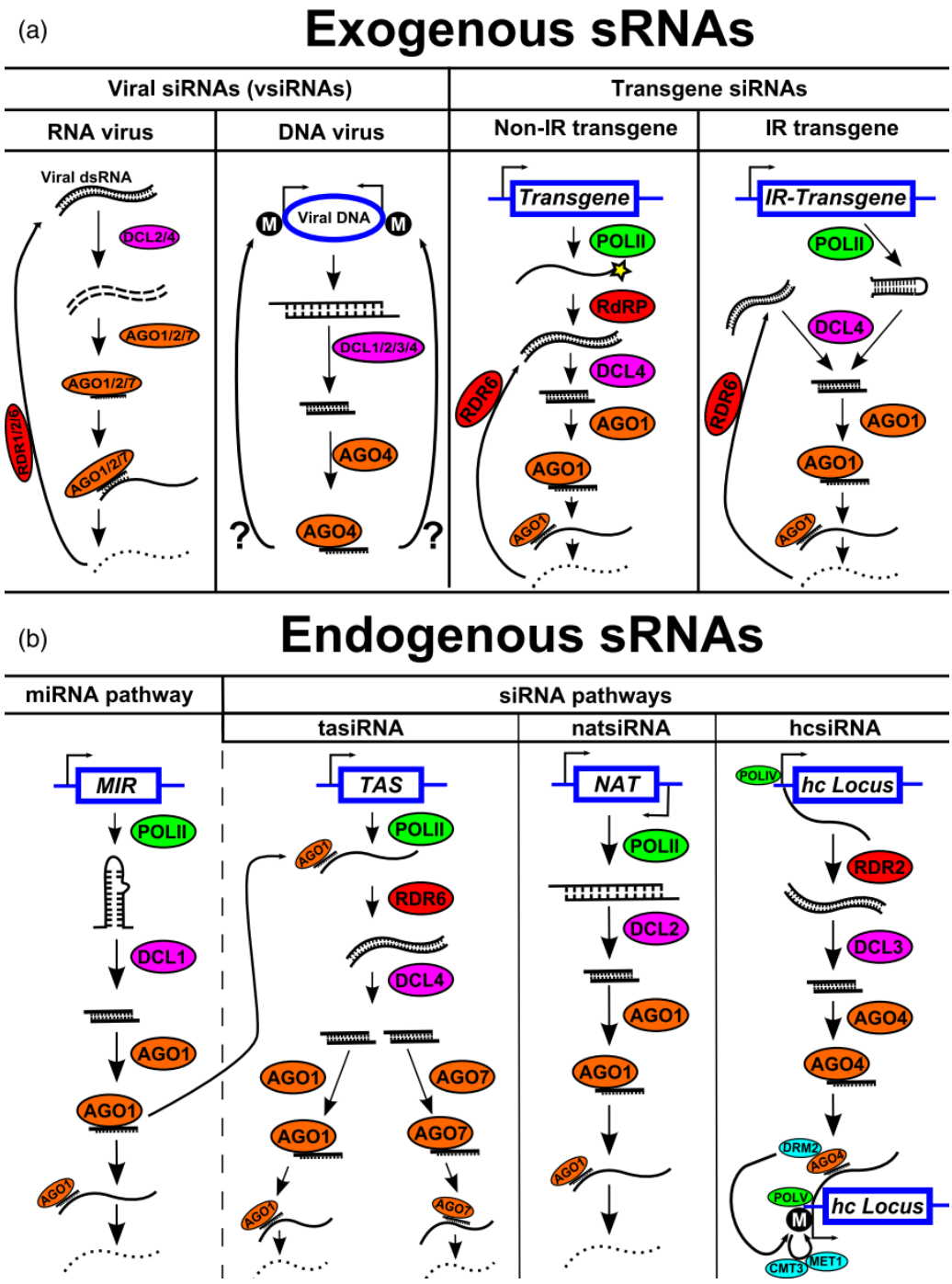

Fig. 1.2 RNA silencing pathways in plants (modified from Pyott and Molnar, 2015).

1.3. Post-transcriptional gene silencing

PTGS is a process which preferentially took place in the cytoplasm to protect plant cell against invading RNAs, such as viruses or transgene derived RNAs (Ratcliff, 1997; Voinnet, 2005). But also, expression of endogenous genes involved in a stress response or plant body development is

affected via PTGS (induced by nat-siRNA, miRNAs, ta-siRNA and some other types of sRNAs; see more in Chapter 1.2.; Bartel et al., 2004; Borsani et al., 2005).

Basic mechanism of mostly 21-22 nt long si/miRNA formation, essential for the next steps in PTGS, was indicated in Chapters 1.1. and 1.2. The moment the siRNA was created, non-guide passenger siRNA* strand removed and guide siRNA strand loaded into an AGO protein, effector RISC complex was established. This executive system ends with mRNA cleavage or protein translation inhibition (Baumberger and Baulcombe, 2005; Brodersen et al., 2008).

In a model plant *A. thaliana*, AGO family involves 10 protein paralogs and DCL family 4 protein paralogs, which have distinct roles in a broad spectrum of RNA silencing pathways (Margis et al., 2006; Hutvagner and Simard, 2008). The existence of multiple, partially redundant PTGS routes contributes to the high robustness of the PTGS process but complicates the characterization of individual pathways (Hoffer et al., 2011). Considering its efficiency, PTGS was exploited as the most commonly used research tool for targeted gene suppression in plants (Watson et al., 2005). The most frequent strategy is the expression of an inverted repeat construct. Its transcripts form a hairpin RNA structure, whose dsRNA part is processed to sRNAs. Those molecules can down-regulate internal or external genes (Eamens et al., 2008).

However, little is know for example how is PTGS initiated *de novo* against elements that are not supposed to produce dsRNA (Parent et al., 2015). It has been also not fully understood how PTGS, its dynamics and reversibility vary when sRNAs are produced from different dsRNA procursors. Many questions were still not answered about interconnection of PTGS and TGS, that is why I come with “few pieces” in this thesis into this huge jigsaw puzzle.

1.4. Chromatin epigenetic modifications

The genetic information in every cell exists as a sequence of nucleotides in a DNA molecule, which is associated with interacting proteins forming together the so-called chromatin. In eukaryotic organism the structural subunit of chromatin is a nucleosome, DNA segment wrapped around a core of eight histone proteins. Epigenetic modifications of histone proteins and DNA represent an additional layer of information that affects the expression of underlying genes (Law and Jacobsen, 2010).

RNA-driven chromatin (DNA) modification was for the first time described in tobacco plants, where the accumulation of transgene derived RNA resulted in sequence-specific DNA methylation (Wassenegger et al., 1994). Few years later, sRNAs were determined as a causal molecules responsible for DNA methylation and histone 3 methylation at lysine 9 (H3K9me; Figure 1.4.2a) leading to the formation of heterochromatin (Hamilton et al., 2002). This type of

heterochromatic mark was then confirmed throughout the eukaryotic kingdoms, like in mice, *C. elegans* or *Drosophila* (for review see Castel and Martienssen, 2013), where unlike plants, DNA methylation is erased every generation (for review see Heard and Martienssen, 2014). On the other hand, histone modifications like acetylation or H3K4me are connected with euchromatic regions (Meyer, 2011). The whole picture of epigenetic chromatin modification and regulation of its structure is much more complicated and beyond the scope of introduction in this thesis.

1.4.1. DNA methylation and demethylation

DNA methylation, a key chromatin epigenetic modification, is evolutionarily ancient biochemical process contributing to regulation of gene expression, genome structure and integrity in Eukaryotes (Law and Jacobsen, 2010; Zemach et al., 2010).

In plants, DNA methylation is usually introduced to cytosines in all sequence contexts – symmetric CG and CHG and asymmetric CHH (where H is A, T or C), leading mostly to the transcriptional repression (Henderson and Jacobsen, 2007). The most abundant form of methyl cytosine (mC) in plants, mCG, is maintained by methyltransferase 1 (MET1), plant homolog of DNMT1 (DNA methyltransferase 1; Jones and Taylor, 1981). This process is closely correlated with DNA replication (Jones et al., 2001). Methylation of non-CG sites is introduced by plant-specific chromomethylases 2/3 (CMT2/3) through a self-reinforcing loop (H3K9me; Chapter 1.4.; Figure 1.4.2a; Du et al., 2015) and by DRM2/1 via the RdDM pathway (Chapter 1.4.2.; Figure 1.4.2a; Zhong et al., 2014). DNA methyltransferases need chromatin remodeling proteins like DDM1 (decrease in DNA methylation 1) and DRD1 (defective in RNA-directed DNA methylation 1) for their proper functioning and access to histones (see also Chapter 1.5.1.; Kanno et al., 2004; Zemach et al., 2013).

Genome-wide analysis of DNA methylation in *A. thaliana* using the bisulphite-Illumina sequencing revealed overall levels of 24% CG, 6.7% CHG and 1.7% CHH methylation. Major part of CG sites is highly methylated (80-100 %) or unmethylated, while CHHs are either unmethylated or methylated at ~10 %. CHG methylation is uniformly distributed at the level of 20-100 % (Cokus et al., 2008). The CG, CHG and CHH methylation occurs together predominantly in repeat-rich pericentromeric heterochromatic regions, concurrently heavily producing 24nt siRNAs (see Chapter 1.4.2.). Differently, almost exclusive CG methylation was detected in about one third of transcribed gene bodies, whereas only ~5 % of genes show methylation within their promoter regions (Zhang et al., 2006; Cokus et al., 2008). In fact, methylation in the promoter arrests the transcription (TGS), while CG methylation in the gene body does not (very often accompanying PTGS; Lister et al., 2008; Lunerová-Bedřichová et al., 2008; Bewick and Schmitz, 2017). In case of transgenes, silencing is usually followed by

methylation of both the promoter and the transcribed protein coding sequence (Fojtova et al., 2003).

Even though in most cases DNA methylation is a stable epigenetic mark, limited levels of methylation are noticed during early stages of plant development (Bouyer et al., 2017). This depletion of methylation can either appear passively, by DNA replication in the absence of functional maintenance methylation routes, or actively, by the elimination of methylated cytosines (Elhamamsy, 2016). In *A. thaliana* DNA glycosylases hold the function of active demethylation – mainly repressor of silencing 1 (ROS1), demeter-like 2/3 (DML2/3) and endosperm-specific demeter (DME; for more details read Law and Jacobsen, 2010). This demethylation has several functions including an important role in TE inactivation during gametogenesis (read more in Chapter 1.4.2. and 1.5.1.; Slotkin et al., 2009; Law and Jacobsen, 2010).

Another possibility how to negatively manipulate DNA methylation is to block MET1 activity by artificial application of methylation-inhibitors like 5-Aza-2´deoxycytidin (AzaC) or zebularine, but this can heavily affect plant growth and development, which is also connected with induction of DNA damage (Fieldes et al., 2005; Baubec et al., 2009; Marfil et al., 2012; Nowicka et al., 2019).

1.4.2. RNA-directed DNA methylation

Regulation of gene expression can be at transcriptional or posttranscriptional level (Chapter 1.3.) and is driven typically by specific size categories of sRNAs – 21-22 nt for PTGS and 24 nt for RdDM and transcriptional gene silencing. In canonical RdDM, which serves primarily for the maintaining of DNA methylation, sRNAs are produced from transcripts of plant specific pol IV (Fig. 1.4.2a; Law et al., 2013; Cuerda-Gil and Slotkin, 2016). Nascent transcript is immediately transcribed into dsRNA by RDR2 (Smith et al., 2007) and processed by DCL3 into one 24 nt siRNA. It is stabilized by 2´*O*-methylation (Li et al., 2005) and guide strand is typically incorporated into AGO4 or AGO6 (Havecker et al., 2010). AGO-carried sRNA then associates with other important proteins at non-protein-coding scaffold transcript of Pol V and manages *de novo* methylation of corresponding loci through domains rearranged methyltransferase 2 (DRM2; Wierzbicki et al., 2008; Matzke and Mosher, 2014). When DNA methylation is settled, heterochromatin can be created by other chromatin modifying enzymes and remodeler complexes (for more details see Matzke and Mosher, 2014).

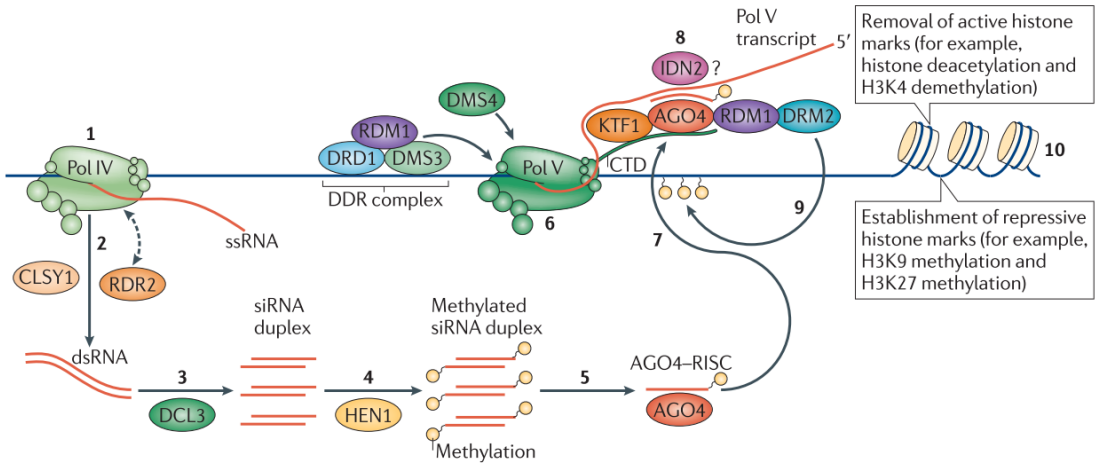

Fig. 1.4.2a Model for the canonical RNA-directed DNA methylation in *Arabidopsis thaliana* (modified from Haag and Pikaard, 2011).

In the past decade, several research groups described alternative non-canonical RdDM pathways which combine other proteins of RNAi machinery and sometimes even routes from PTGS. Mostly they does not need Pol IV or RDR2 for production of siRNAs (Herr et al., 2005; Pontier et al., 2012; Nuthikattu et al., 2013). i) One possibility is to use hairpin Pol II-derived transcripts of inverted repeats or microRNA genes and process them by DCL3 into 24 nt sRNAs or by DCL 2/4 into 21-22 nt sRNAs (without involvement of RDRs; see Fig. 1.4.2b; Cuerda-Gil and Slotkin, 2016; Panda et al., 2016). This alternative way of RdDM can serve for some TE loci (Slotkin et al., 2005). ii) Another possibility is coupling PTGS to RdDM through the creation of secondary 21-22 nt sRNAs by the activity of AGO1, RDR6 and SGS3 (Cuerda-Gil and Slotkin, 2016; Lee and Carroll, 2018). Typical target of such a pathway could be *TAS* loci (Wu et al., 2012). Detailed overview of all possible RdDM routes is reviewed in Matzke and Mosher (2014) and Cuerda-Gil and Slotkin (2016).

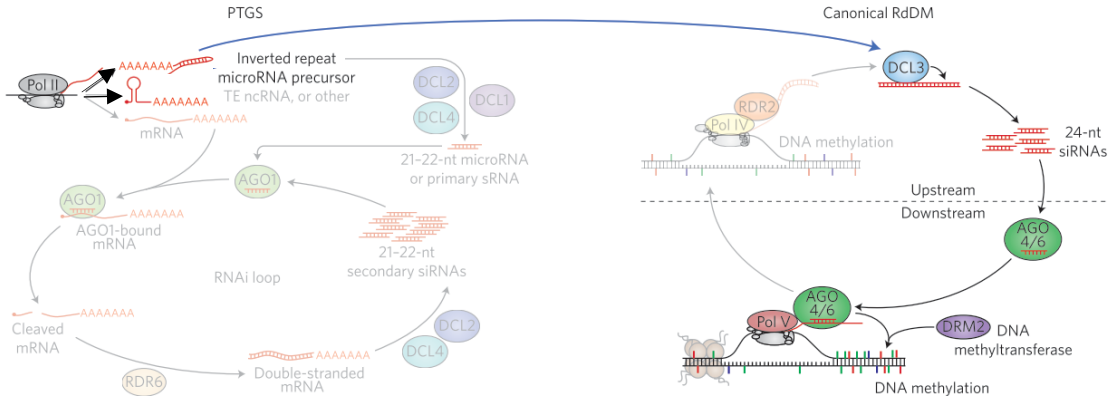

Fig. 1.4.2b Model for an alternative RdDM pathway (modified from Guerda-Gill and Slotkin 2016).

1.5. sRNA movement in plants

Remarkably, some sRNAs can participate in cellular processes at different sites other than their origin. They can be transported from cell-to-cell and travel over long distance. This systemic character of silencing signal represented by nucleic acids was assumed in studies with plants exhibiting silencing (PTGS or TGS) of transgenic reporters (Palauqui et al., 1997; Voinnet and Baulcombe, 1997), even though some evidences were known from earlier studies with petunia (Napoli et al., 1990; Jorgensen, 1995). Historical observations of mobile silencing prefigure the cardinal discovery that double-stranded RNA could lead to sequence-specific silencing (Fire et al., 1998). At the very same year Voinnet et al. (1998) showed by sophisticated grafting experiments that the silencing signal could by transported to sink organs by phloem vasculature. This hypothesis was also supported by tests with cadmium inhibition of phloem transport (Ghoshroy et al., 1998). More recently, a much wider world of sRNAs of external provenance was revealed in cross-kingdom interactions. Cross-kingdom RNAi is the process in which sRNA regulation of gene expression is induced between two individuals of unrelated species coming from different kingdoms, like a plant host and its interacting pest/pathogen/parasite/mutualistic symbiont (Weiberg and Jin, 2015). Such an interaction can occur in both ways – i) sRNAs produced for example by parasites and pathogens can be translocated into plant host cells and trigger gene silencing of host defense genes (Weiberg et al., 2013) or ii) in a phenomenon called HIGS (host-induced gene silencing) plant-produced RNAi signal can trigger silencing of e.g. some essential pathogen gene (Nunes and Dean, 2012), in case of viruses we refer to VIGS (virus-induced gene silencing; Lu et al., 2003). Very recently Cai et al. (2018) showed that this type of sRNA-mediated communication between *A. thaliana* and pathogen *Botrytis cinerea* is done by the secretion of exosome-like extracellular vesicles.

Generally, when the silencing signal is established in a plant cell, it can symplastically spread as siRNA (Dunoyer et al., 2010; Zhang et al., 2014) or miRNA (Juarez et al., 2004) through plasmodesmata (PD) for a **short range**, presumably by passive diffusion into small number of surrounding cells (as shown in Fig. 1.5a; Himber et al., 2003; Pyott and Molnar, 2015). In addition to the short-range movement of RNA silencing, a **long-range** mechanism uses also PDs but needs an amplification of sRNAs by secondary sRNA synthesis (for more information read Chapter 1.2. and see Fig. 1.5b). Difference between short- and long-range cell-cell silencing depends on the biogenesis pathways producing the sRNAs (Felippes et al., 2011). **Systemic** transport of silencing signal to distant tissues is dependent on phloem vasculature (illustrated in Fig. 1.5c; Voinnet et al., 1998). All classes of RNA molecules, including 21-24 nt sRNAs and miRNAs were detected in phloem (Yoo et al., 2004; Buhtz et al., 2008; Molnar et al., 2010; Kehr and Kragler, 2018). It was shown in study of Palauqui et al. (1997), that silencing signal moved from lower (older) parts mostly to the upper, younger tissues. Although our knowledge about the intercellular siRNA and miRNA transport within plants is growing, detailed mechanism and key players remain mostly ambiguous (Kehr and Kragler, 2018). Critical step can be for example up- and unloading of sRNAs through apoplast in apoplastic phloem loaders.

Transport of sRNAs to symplastically isolated cells, likely takes place in a different way than by PD. Simultaneously biochemical processes are in these "special regions" highly regulated from outside during plant development or stresses. Such examples could be stomata guard cells (Voinnet et al., 1998) or a vegetative nucleus and generative cells in mature pollen grains (Slotkin et al., 2009). In the following Chapter 1.5.1., I focused on sRNA movement in *A. thaliana* pollen grains, which is relatively well characterized.

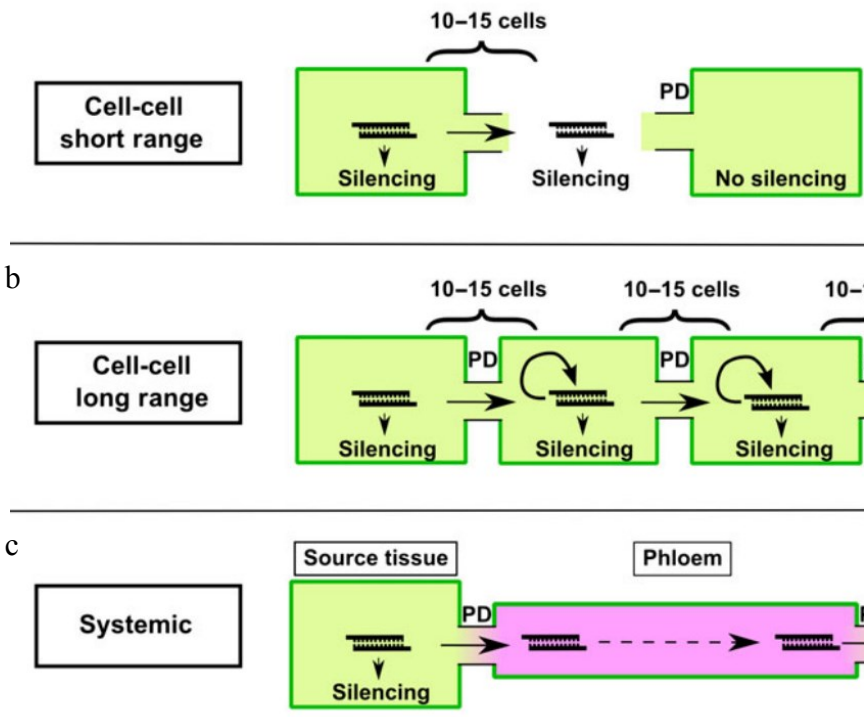

Fig. 1.5 All kinds of traditional sRNAs movements in plant body (Pyott and Molnar, 2015).

1.5.1. Mobile sRNAs during male gametogenesis

Endogenous siRNAs play also crucial regulatory role during gamete development in plants. I will concentrate here only on description of sRNA action in *A. thaliana* sperm cell, thought analogical process has been reported in female gametes (Olmedo-Monfil et al., 2010). Mature pollen grain at the end of male gametogenesis (for more details read McCormick, 1993) consists of three cells – larger (vegetative) cell encapsulating 2 smaller (generative) sperm cells (as shown in Fig. 1.5.1). Vegetative cell fate is particularly to support and deliver both sperm cells for fertilization of the egg cell and the central cell of the female gametophyte. Another essential purpose was discovered by Slotkin et al. in 2009 as an ingenious system for sperm cell DNA protection against the mutagenic activity of TEs. Downregulation of chromatin remodeling ATPase DDM1 in vegetative nucleus leads to the reactivation of many genomic loci (containing typically TEs) and subsequent massive siRNAs production. These siRNAs then presumably move to sperm cells and assure silencing of the same TEs in their genomic DNA by small RNA-directed DNA methylation (RdDM, Chapter 1.4.2a.; Slotkin et al., 2009). This important tool maintains intact TEs in the next generation preventing deleterious effects of their transposition in the germ line (Slotkin et al.,

2009; Pyott and Molnar, 2015). The main uncertainty of understanding this process stays in visualization and detection of particular sRNAs and their movement.

How these siRNAs can overcome the plasmatic membrane of the generative cell isolating cytosols of those two separated cells in pollen grain remains unclear. Some authors had considered long cytoplasmic extensions (McCormick, 2004), which were not confirmed later. One might expect even endo/exocytosis (Cai et al., 2018), that works for animal cells (summarized in Jose, 2015). Nevertheless, the discovery of a transmembrane protein indispensable for systemic RNA silencing in animal body of *Caenorhabditis elegans* (Winston et al., 2002), suggested that special RNA transporters should be also taken into account. The system of sRNA movement between animal cells, which are not interconnected by cytoplasmic "sleeves", opened the huge field of research focused on manipulation of gene expression by externally supplied RNAs (for example Feinberg and Hunter, 2003; Mon et al., 2012). However, to test the hypothesis, that sRNAs can move between symplasticaly isolated cells with the help of protein transporters, will be a challenge in plant cells generally interconnected by PD.

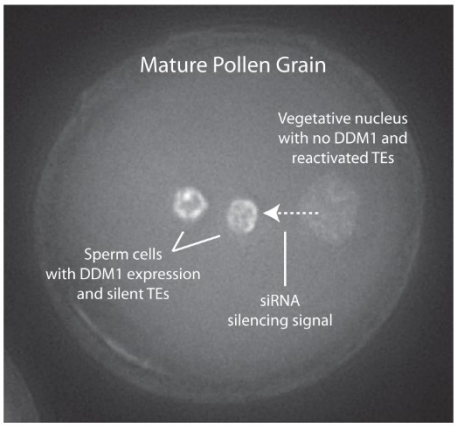

Fig. 1.5.1 Model of TE derived siRNA movement from vegetative nucleus to sperm cell in mature pollen grain (Slotkin et al., 2009).

1.6. sRNA movement in *Caenorhabditis elegans*

The huge breakthrough in the RNAi field was done in dsRNA delivery studies with *C. elegans* (Fire et al., 1998; Timmons and Fire, 1998). Nevertheless, the systemic nature of RNAi, where dsRNA for example injected into one tissue lead to the silencing in other tissues, remained mysterious until the discovery of very first candidate gene, *SID-1*, in the systemic RNAi defective screen by Winston et al. in 2002. Since then, the world of animal systemic RNAi has been uncovered with increasing intensity.

SID-1 was shown to be a transmembrane channel with 11 predicted transmembrane domains (Fig. 1.6a). It was documented to be crucial for dsRNA import into the cytoplasm (as illustrated in Fig. 1.6b), but not required for the export of mobile dsRNA from muscles or intestinal cells of *C. elegans* (Jose et al., 2009; Shih and Hunter, 2011; Whangbo et al., 2017). Its homologues were found in all yet sequenced vertebrates, many invertebrates and even in phylogenetically unrelated amoeba *Dictyostelium discoideum* (Fig. 9.1.; Winston et al., 2002; Feinberg and Hunter, 2003). Functional conservation has been approved by the expression of *C. elegans SID-1* in different species (Feinberg and Hunter, 2003; Xu et al., 2013), enabling a passive uptake of dsRNA (Feinberg and Hunter, 2003). SID-1 is also fundamental and absolutely required for inheritance of silencing by dsRNA (Wang and Hunter, 2017), although the precise molecular function and biochemical activity remain pending (Liberman et al., 2019).

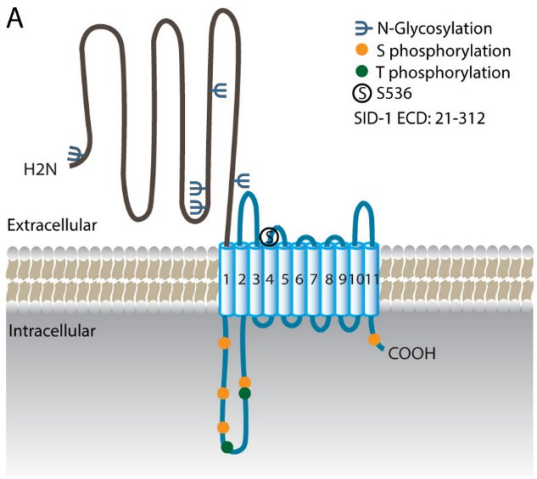

Fig. 1.6a SID-1 predicted topological model with post-translational modifications. It has rich extracellular domain (~300 AA) and highlighted key residue Ser-536 (Li et al., 2015).

Another player involved in the systemic RNAi in *C. elegans* is SID-2. Analysis of *sid-2 (qt13)* mutant worms showed full sensitivity to systemic RNAi initiated by microinjection or transgenic expression of dsRNA targeting somatic and germ-line-expressed genes, but at the same time they were resistant to feeding RNAi (orally administrated dsRNA; Winston et al., 2007). Thus, SID-2 is required likely for endocytosis-mediated uptake of silencing information (from environment into gut cells) but not for RNAi spreading among cells (Winston et al., 2007; Rocheleau, 2012). SID-2 works as a single-pass transmembrane receptor protein with low sequence conservation (unlike SID-1) even within other nematodes (Fig. 1.6b; McEwan et al., 2012; Jose, 2015).

The screen of Jose et al. (2012) for RNAi defective worms uncovered SID-3 protein as a conserved tyrosine kinase required for the efficient import of dsRNA. Without SID-3, cells perform RNA silencing well but the rate of dsRNA import is poor (Jose et al., 2012). In the same year another single-pass transmembrane protein SID-5 was also discovered that partially colocalizes with endosomes and could play some role in the vesicle transport (Hinas et al., 2012). A model of intercellular spreading of RNAi in *C. elegans* mediated by SIDs factors is described in the Fig. 1.6b. More information about interconnections between RNAi and transgenerational epigenetic inheritance is summarized in Minkina and Hunter, 2018.

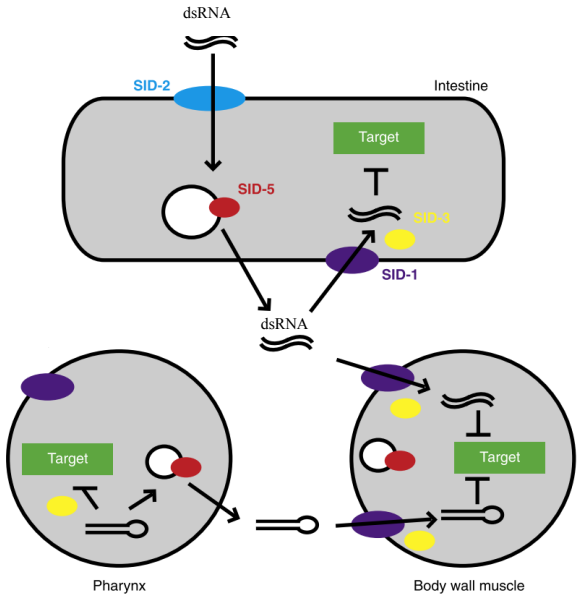

Fig. 1.6b Model of RNAi in *C. elegans* showing involvement of SID proteins (modified from Rocheleau 2012).

2. MAIN AIMS AND HYPOTHESIS

Research presented in this thesis aimed to shed light on several not deeply understood mechanisms of RNAi with possible impact not only to basic, but especially to applied research. Combination of different plant model organisms helps us to use their benefits for achieving our goals described below.

(I) Reactivation of silenced transgenes in *Solanum tuberosum* plants at the whole plant level:

Hypothesis: *Can we restore the expression of transcriptionally silenced transgenes in plants? Are there any factors which can induce silencing of transgenes?*

a. reactivation of transgene expression after its spontaneous silencing at TGS level;
b. determination of the methylation and the transcription profile of restored transgenes;
c. discovery of factors which can lead to the re/silencing of introduced transgenes;
d. investigation of the transition between PTGS and TGS and subsequent spread of methylation within T-DNA.

(II) PTGS and TGS dynamics and their interconnections:

Hypothesis: *What are the effects of different silencers on PTGS dynamics?*

What is the course and the dynamics of early stages of de novo DNA methylation in TGS?

What type of sRNAs are involved in promoter RdDM and how rapidly they arise?

a. assessment of the methylation pattern accompanying PTGS;
b. appraisal of the methylation character in TGS;
c. pinpointing the transcription level of targeted genes;
d. investigation of siRNA origin, level and dynamics.

(III) Uncovering of plant sRNA transporters:

Hypothesis: *Do plants have special sRNA transporters analogical to animal SID-1?*

a. selection of plant candidate genes, homological to animal dsRNA transporter;
b. preparation of plant mutant material with modified expression of such genes;
c. evaluation of phenotype changes in mutants and investigation of sRNA transport between symplasticaly isolated cells.

3. MATERIALS AND METHODS[1]

3.1. Plant material, cultivation conditions and transformation

Potato plants (*Solanum tuberosum* L. cv. Désirée) were cultivated *in vitro* on the LS medium (Linsmaier and Skoog, 1965), containing 3% (w/v) sucrose in a cultivation room under long-day photoperiod (16 h light, 8 h dark; 23 °C; PPFD approximately 200 μmol m^{-2} s^{-1}). Sub-cultivation interval was 4-6 weeks. Transformation of leaf explants taken from 4-week-old plants was performed according to Dietze et al. (1995) using *Agrobacterium tumefaciens* strain C58C1RifR carrying a helper plasmid *pGV2260* (Deblaere et al., 1985) and modified binary vector *pCP60* (Fig. 1.3a-a; Bolte et al., 2004; Nocarova and Fischer, 2009). T-DNA introduced into plants consists of two genes – *CaMV35S*::*rsGFP* and *Pnos*::*nptII* (read more in Tyč et al., 2017). For this work two lines that spontaneously silenced expression of both transgenes (R17 with a single insertion and R28 with multiple insertions) were selected from the collection described in Nocarova et al. (2010).

The tobacco BY-2 cell line (*Nicotiana tabacum* L. cv. Bright Yellow; Nagata et al., 1992) was cultivated in the modified MS medium (Murashige and Skoog, 1962). MS salts (Merck) were supplemented with 200 mg/L K_2HPO_4, 100 mg/L myo-Inositol, 3% (w/v) sucrose, 1 mg/L vitamin B1, and 1μM 2,4-Dichlorophenoxyacetic acid, pH adjusted to 5.8 with 1M KOH. Cultures were kept at 27 °C in 100mL Erlenmeyer flasks on an orbital shaker IKA KS501 at 110 rpm (IKA Labortechnik) in a darkroom. The cell lines were normally sub-cultured weekly by 1 mL into 30 mL fresh media, "continually exponential cultures" were sub-cultured every 3–4 days by 1.5 mL. The transformation protocol was used as reported by Dvořáková et al. (2012). Non-homogeneous cultures (in the respect of fluorescent protein fluorescence) were sub-cloned according to Nocarova and Fischer (2009) before starting the experiments. The list of all prepared transgenic lines is presented in Table 3.1 below.

[1] This chapter describes in detail particularly the methods and plant material which were not include in attached joint publications avoiding repetition.

Tab. 3.1 The list of used transgenic BY-2 lines, their genetic background and main purpose.

name	binary vector	gene sequence 1[1]	gene sequence 2[1]	selection marker	Figure	reference
GREEN1	*pCP60*	*rsGFP*		*KanR*	3.1a-a	[1]
IR8C[2]	*pCP60* *pER8*	*rsGFP* *IR-rsGFP*		*KanR* *HygR*	3.1a-a 3.1a-b	[2]
GRED1	*pGREEN0129*	*rsGFP*	*mCherry*	*HygR*	3.1a-c	this thesis
GRED2	*pGREEN0129*	*rsGFP*	*TagRFP*	*HygR*	3.1a-d	this thesis
GRSA[2]	*pGREEN0129* *pGREEN0029*	*rsGFP* *AtSAG18*	*TagRFP*	*HygR* *KanR*	3.1a-d 3.1b-a	this thesis
GRCE[2]	*pGREEN0129* *pGREEN0029*	*rsGFP* *CeSID-1*	*TagRFP*	*HygR* *KanR*	3.1b-d 3.1b-b	this thesis
SAF	*pGREEN0029*	*AtSAG18* *E-GFP* fusion		*KanR*	3.1b-c	this thesis
CEF	*pGREEN0029*	*CeSID-1* *E-GFP* fusion		*KanR*	3.1b-d	this thesis

References: [1] Nocarova and Fischer, 2009
[2] (Čermák et al. submitted)

Notes: [1] sequence in additional data 9.5.
[2] super-transformed

Arabidopsis thaliana ecotype Columbia (Col-0) plants were grown in Jiffy soil pellets under the same conditions as *S. tuberosum* plants. For the hairy root length analysis, the seedlings were grown *in vitro* on vertical agar plates (12 cm side) with 0.2x MS medium (MS/5) supplemented with 1% (w/v) sucrose and 1% (w/v) agar. Plates with seeds were stratified at 4 °C for 48 h and then cultured as mentioned above. *A. thaliana* genotypes used in this study were Col-0 (wt), *sag18* (SALK_022062), *aphc* (SALK_003875) obtained from NASC T-DNA mutant collection (Scholl et al., 2000).

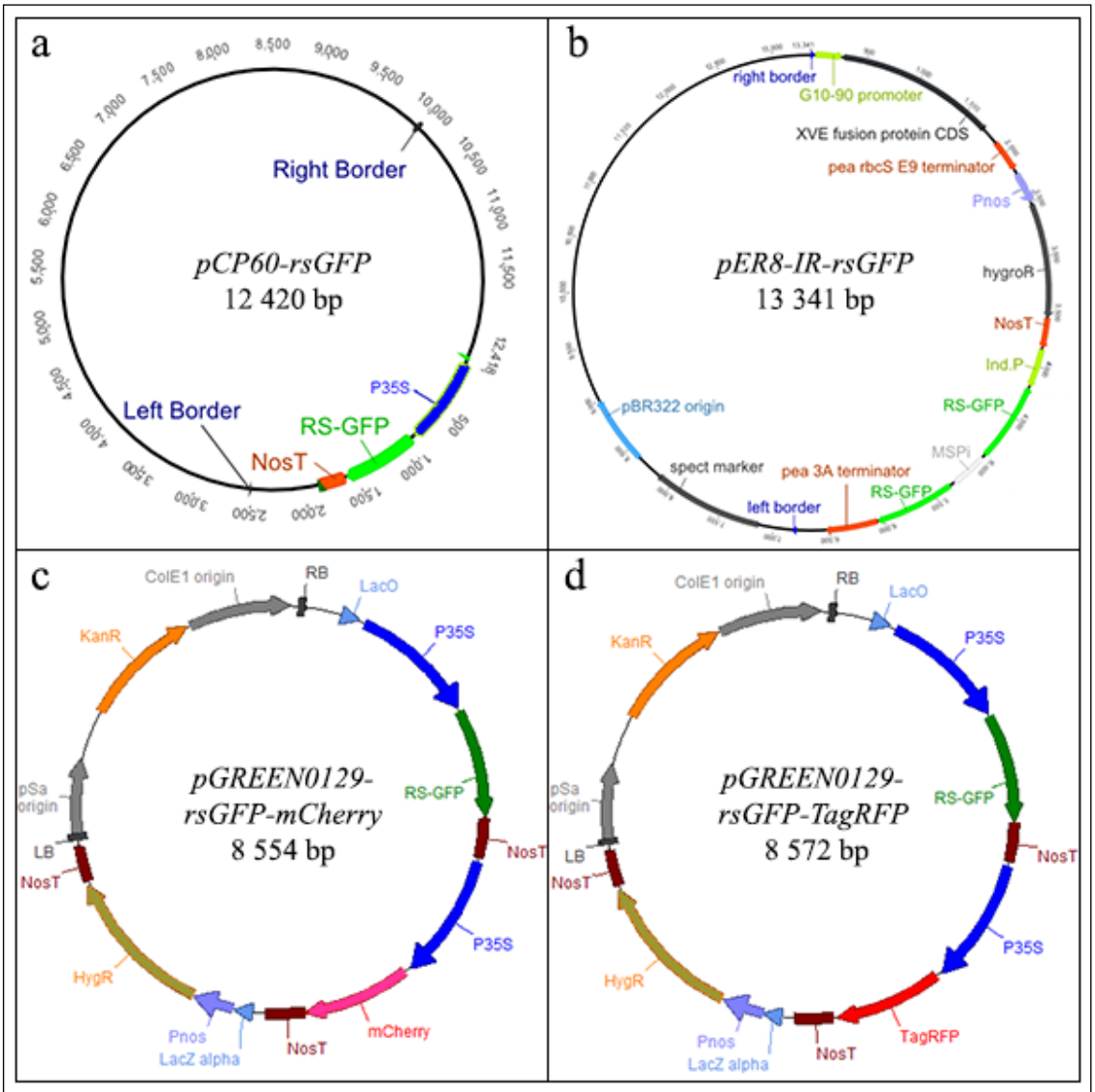

Fig. 3.1a Maps of binary vectors 1/2 used (**a-d**) and prepared (**c**, **d**) during this thesis. Figures for (**a**, **b**) were kindly provided by Adéla Přibylová. Processed by ApE-A plasmid Editor v2.0.61.

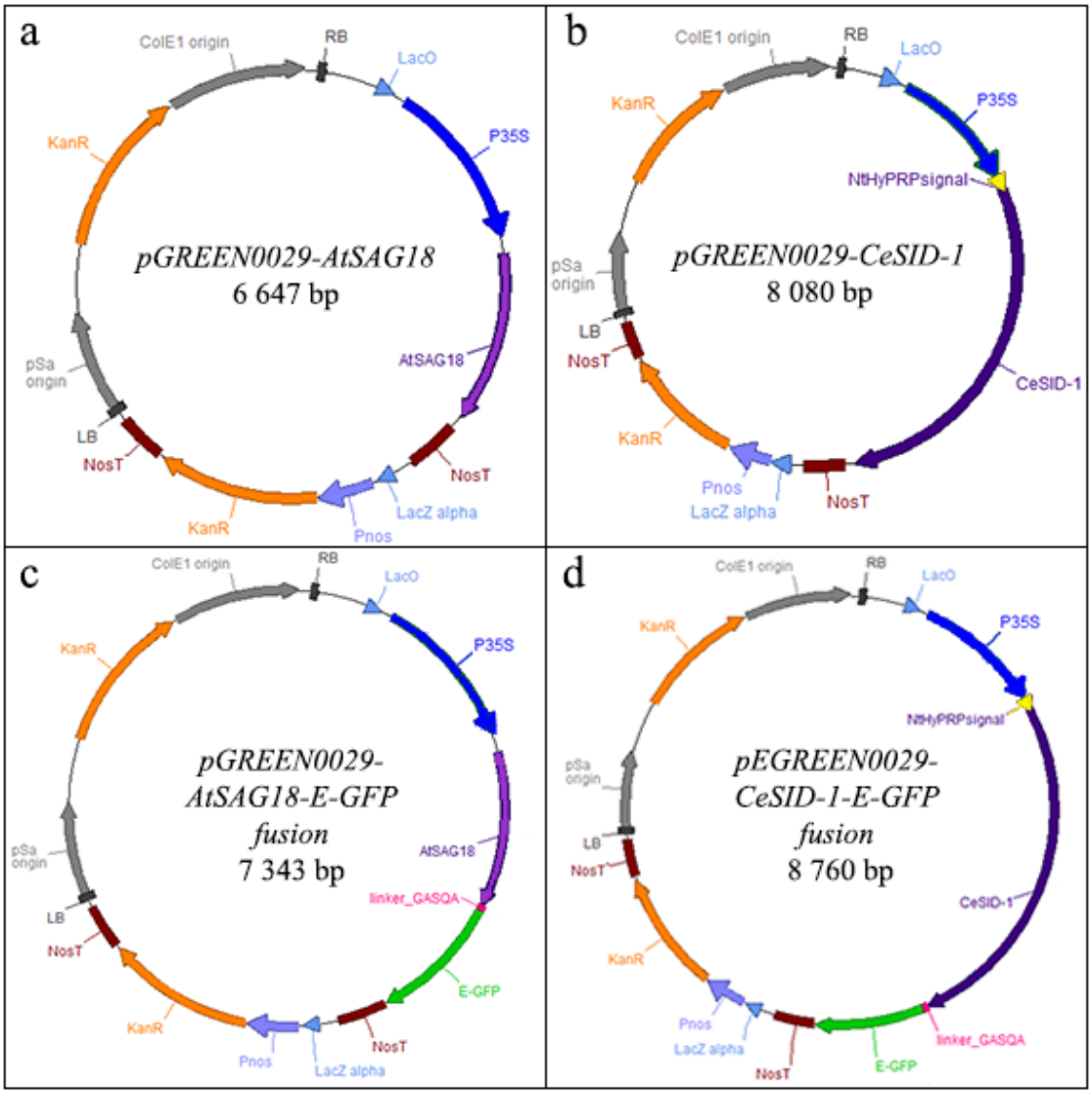

Fig. 3.1b Maps of binary vectors 2/2 used and prepared during this thesis. Processed by ApE-A plasmid Editor v2.0.61.

3.2. Transgene reactivation

Reactivation of transgene expression via *de novo* regeneration is based on the *S. tuberosum* transformation protocol from Dietze et al. (1995). Leaf explants from 4-week-old leaves from selected *in vitro* plants with silenced transgene expression were grown for three days on the callus-inducing medium in 6 cm plastic Petri dishes. Afterwards, the leaf segments were transferred to the same medium but supplemented with freshly added 10 μM AzaC and cultured for four days (Sigma-Aldrich; 20 mM stock in filter-sterilized water solution, stored at -20 °C few weeks). The explants were subsequently transferred every two weeks to the shoot-inducing medium supplemented only with kanamycin (50 mg/l). Newly developed shoots were then cultivated in LS medium and selected lines were assessed for restored transgene expression at protein (Chapter 3.3) or transcript levels (Chapter 3.4). For more details, please, see Tyč et al. (2017).

3.3. Fluorescence analysis

Methods for fluorescence assessment differed depending on the plant material. In whole plants, reporter genes were examined as described in Tyč et al. (2017), using fluorescent microscopes Olympus Provis AX70 and Olympus BX51. Pictures were processed with Lucia software (Laboratory Imaging), Zoner Photo Studio (Zoner software) or Helicon Focus (Helicon Soft Ltd).

Fluorescence of tobacco BY-2 cell line in calli was also analyzed by microscopes or as described in (Čermák et al. submitted), measured on photo-documenting system G:BOX (SynGene). Images were processed using software NIS-Elements 3.10 (Laboratory Imaging) with measuring all pixels from each calli, excluding non-homogenous ones and subtracting background of wt callus. Statistical analyses were done by Wilcoxon signed-rank test and Wilcoxon rank-sum test in R 3.4.4.

Fluorescence of tobacco BY-2 cells in suspension cultures was analyzed at the single-cell level using flow-cytometer LSR II (BD biosciences). Protoplasts for flow-cytometry were prepared as described in Chapter 3.8. and in Přibylová et al. (2019). On average about 14000 cells were measured per sample. The data were processed by Flowing Software or FlowJo vX.0.7 with the selection of live protoplasts as described in (Klíma et al., 2019). BY-2 cell lines with the GFP protein fusions (i.e. SAF and CEF lines; see Tab. 3.1) were analyzed by confocal microscopes Leica TCS SP2 and Zeiss LSM 880.

3.4. Transcription analysis

Transcript levels of selected genes were evaluated by RT-qPCR. RNA was isolated from 100 mg of frozen sample using the phenol-chloroform isolation protocol (White and Kaper., 1989) or RNeasy® Plant Mini Kit (QIAGEN; Tyč et al., 2017). The quantity and quality were checked by the gel electrophoresis and NanoDrop 2000. cDNA preparation and transcripts quantification of selected genes were performed as described in Bustin et al. (2009) and Tyč et al. (2017). Shortly: RNA was transcribed using RevertAid Reverse Transcriptase (Thermo Fisher Scientific) and oligoT23 primer. Determination of transcripts was done on a LightCycler 480 (Roche) using the iQ TM SYBR Green Supermix (BioRad,) with primer pairs for TagRFP (5´-GAGGGAAAGCCATACGAGGG-3´ and 5´-AAGTGGTAACCCTCTCCCATG-3´), EF1α (5´-TACTGCACTGTGATTGATGCT-3´ and 5´-AGCAAATCATTTGCTTGACAC-3´) and rsGFP (5´-GAGACACCCTCGTCAACAGG-3´ and 5´-TGGTCTGCTAGTTGAACGCTT-3´) or primers listed in Attachments 10.2. (Tyč et al., 2017), in Attachments 10.6. (Přibylová et al., 2019) and Attachments 10.4. (Čermák et al. submitted). The specificity of the RT-qPCR was performed bymelting curve analysis (using the LightCycler 480 software). The resulting data were processed by LinRegPCR software (Ramakers et al., 2003) and MS Excel 2016. Calculated transcript concentrations were normalized to the *EF1α* or *TagRFP* (hereinafter *RFP*) transcript levels. For statistical analysis, we used one-way ANOVA.

3.5. DNA methylation analysis

The method for DNA methylation analysis was described in detail in (Tyč et al., 2017). In brief, the genomic DNA of analyzed plants was extracted by the DNeasy Plant Mini Kit (Qiagen), *Eco*RI digested, purified by phenol-chloroform and modified by EpiTect Bisulfite Kit (Qiagen). Selected regions of interest were PCR amplified with primer pairs listed in Attachments 10.2. (Tyč et al., 2017), Attachments 10.6. (Přibylová et al., 2019) and Attachments 10.4. (Čermák et al. submitted). The PCR products were cloned into *pDrive* vector (QIAGEN PCR Cloning Kit) and 6-12 clones per sample were sequenced and analyzed in MS Excel 2016 and web-based tool pKismeth (Gruntman et al. 2008). For statistical analysis, we used two-sample *t* test (Tyč et al., 2017) or Wilcoxon rank-sum test in R 3.4.4. (Čermák et al. submitted).

3.6. sRNA analysis

Analysis of sRNAs was described in depth in Přibylová et al. (2019). sRNA library was prepared using the combinatorial probe-anchor synthesis (cPAS)-based BGISEQ-500 sequencing platform (BGI, China), giving highly reproducible results, comparable with other NGS platforms

(Fehlmann et al., 2016). Briefly, RNA was extracted from 100 mg (fresh weight) sample using RNeasy Plant Mini Kit (Qiagen) and checked for quantity and quality. A fraction of 18–45 nt long sRNAs were recovered from 15% urea-polyacrylamide gel. Extracted sRNAs were supplied with adaptors, transcribed to cDNA and circularized those proper ones, creating DNA nanoballs needed for sequencing. Acquired sRNA library was processed in the software Geneious 9.1.8 (Biomatters) and MS Excel 2016. Only siRNAs matching with the respective T-DNA sequence were used for further analyses.

3.7. Preparation of sRNA solution for BY-2 treatment

BY-2 cell line IR8C (Tab 3.1), was obtained by the super-transformation of two T-DNAs – i) *CaMV35S*::*rsGFP* and ii) rs*GFP* inverted repeat controlled by the VGE system allowing for *β*-estradiol (hereinafter estradiol or ESTR) inducible expression (Zuo et al., 2000; Čermák et al. subbmited). Suspension culture of IR8C was kept in the exponential phase and induced by adding of 2 μM estradiol in DMSO (Sigma-Aldrich) to induce production of high levels of sRNAs against *rsGFP* (hereinafter *GFP*). As a control we treated IR8C only with DMSO. 72 h after the treatment, 100 mg (fresh weight) aliquots of filtrated cells (Nalgene filter) were harvested and stored in -80 °C. RNA was isolated from samples treated by estradiol using phenol-chloroform extraction (White and Kaper, 1989). The quantity and quality were checked by the gel electrophoresis and NanoDrop 2000 (Thermo Fisher Scientific). Quaternary of samples were taken together. One half of samples was enriched for low molecular weight (LMW) RNA fraction by 20% (w/v) PEG 8000 and 2M NaCl precipitation (Rosas-Cárdenas et al., 2011). Both halves were taken together creating the mixture of total RNA and LMW fraction with approximate RNA concentration 2.5 μg/μl. Considering its usage for BY-2 protoplast treatment and RNA instability, 0.9M d-mannitol and RiboLock™ RNase inhibitor (Thermo Fisher Scientific) were added tocreate a final solution – 1.25 μg/μl RNA, 0.45M d-mannitol and 1 U/μl of RiboLock. RNA solution was used for BY-2 protoplast treatment to indirectly observe presumed movement of sRNAs through membrane channels into the recipient cell cytosol.

3.8. Treatment of BY-2 protoplasts with sRNA solution

Continually exponential suspension cultures of BY-2 cell line GRED213, GRSA33 and GRCE1 were selected and cultivated under conditions described above (Chapter 3.1.; Table 3.1). Sterile protoplast were prepared according to (Přibylová et al., 2019), briefly – 1.5 mL (GRCE1) or 2 mL (GRSA33 and GRED213) of the cell culture were taken and drained from the medium. 3 mL of protoplast enzyme mixture in 0.45M d-mannitol were added to the cells in the sterile 6-well

cell-culture plate in a dark for 3 h at 26 °C with gentle shaking on an orbital shaker. The mixture was enriched with MS medium with 0.4M sucrose after the incubation. Protoplasts were gently centrifuged (200 RCF, 5 min) without braking. 100 µL of protoplasts from each variant was taken and frozen as a point 0. The rest of protoplasts was divided into four samples per variant – and treated/untreated with 100 µg of sRNAs solution prepared as described in previous Chapter 3.6. 100 µL of protoplasts from each variant was taken and frozen after 6 and 24 h of their cultivation in conditions for the 6-well plate described above. Treated protoplasts were assessed by RT-qPCR for *EF1α*, *RFP* and *GFP* transcript levels. For statistical analysis, we used one-way ANOVA with post hoc Tukey's HSD test.

3.9. Assessment of growth and yield parameters

A. thaliana plants (60 together) of different genotypes (wt, *sag18 aphc*) were grown in Jiffy pellets (1 plant per pellet), randomly distributed in trays under the conditions described before. The maximal stem length of each plant was measured six weeks after germination. Other parameters were evaluated six weeks later, when the plants were completely desiccated. The aboveground part of plants was harvested and completely dried. Shoots and seeds were separated for each plant using certified sieve. The weight of both parameters was measured and calculated together with other parameters for each plant in MS Excel 2016. Statistics was done in R 3.4.4. using linear models.

3.10. Hairy root measurement

Surface-sterilized seeds of *A. thaliana* wt, *sag18*, *aphc*, *sag18 aphc* genotypes were sowed in one line approximately 3 cm from an edge of a plate (MS/5) and 0.75 mm from each other, one plate per genotype. Plates were kept for 2 days in a fridge at 4 °C, then moved to cultivation room and grown under conditions described in Chapter 3.1 in slanting position, seeds up. After 4-6 days, when the root system was in average length 15 mm, 12-15 rooting plants were documented on stereomicroscope SZX7s (Olympus) equipped with camera EOS 60D (Canon). Pictures were analyzed using NIS-Elements 3.10 (Laboratory Imaging, CZ) and all hairy roots were measured and calculated from the hypocotyl till the tip. Data were processed using MS Excel 2016 and statistically assessed in R 3.4.4. using linear mixed-effects model, the differences within significant terms and interactions were post-hoc tested by Tukey's HSD pairwise comparison of least-square means according to Lenth (2016).

3.11. *Arabidopsis thaliana* infection by *Botrytis cinerea*

Five-week-old plants of wt, *sag18*, *aphc* and *sag18 aphc* genotypes were treated with 6-µl drops containing spores of *Botrytis cinerea* strain BMM ($5x10^4$ spores/mL in PDB medium) by applying one drop on one leaf (Kroumanová et al., 2019). Together four leaves of similar developmental stage per one plant were infected. Plants were kept for 24 h in a high humidity dark box in laboratory conditions (25 °C) and then transferred into the cultivation room inside of high humidity transparent box (same conditions as for *S. tuberosum*, except ten-times lower irradiance). Relative lesion sizes of 20 leaves per variant were measured at 3 days after the infection. Statistics was done in R 3.4.4. using linear model.

3.12. Transpiration rate and stomatal conductance analyzes

Two different methods were used to measure transpiration rate of *A. thaliana* plants. The first method was derived from an old gravimetric method of Ivanov (1918). Leaves of four-week-old plants were measured by weighing every 2 minutes. Leaves of similar developmental stage (1 per plant) were gradually detached one after another from 3-6 plants, exposed to intensive light (table-lamp) and weighed for 12 minutes to monitor the water loss by transpiration through plasmodesmata. The cut was done by scissors the same way to minimize differences between samples in water loss by the wound. Data were processed using MS Excel 2016 and statistically assessed in R 3.4.4. using linear mixed-effects model, the differences within significant terms and interactions were post-hoc tested by Tukey's HSD pairwise comparison of least-square means according to Lenth (2016).

Second method used photosynthesis measuring system TPS-1 (PP Systems), which passes a measured flow of air over a leaf sealed into a chamber. The TPS-1 first analyzes the CO_2 and H_2O in the air going to the cuvette and then in the air leaving the cuvette. From the flow rate and the change in the CO_2 and H_2O concentrations, the transpiration rate of water and stomatal conductance are determined. The average leaf area for the measurement in the TPS-1 chamber was calculated as 2.4 cm^2. Leaves at similar developmental stage (1 per plant) were gradually detached from four-week-old well-watered plants (11 per variant), put into the measuring chamber and exposed to an intensive light (table-lamp). Data for the stomatal conductance and the transpiration rate were collected and dataset analyzed using MS Excel 2016 and statistically assessed in R 3.4.4. using linear mixed-effect model with Poisson distribution.

4. SUMMARY OF PUBLISHED AND UNPUBLISHED RESULTS

4.1. Publication 1 – Tyč et al., 2017, published

5-Azacytidine mediated reactivation of silenced transgenes in potato (*Solanum tuberosum*) at the whole plant level

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Original research article, *Plant Cell Reports* 36:8 (IF_{2018} = 3.499)

Transgenesis of plant genomes during either basic or applied research is always accompanied by negatively accepted phenomenon of spontaneous transgene silencing. This leads to the PTGS accompanied by possible cytosine methylation of target sequences and transcriptional inactivation of introduced reporter gene/genes even years after the transformation event (Nocarova et al., 2010). Such a transgenic well characterized plant is basically useless for other research. In this study, we focused on finding of possibility of restoration the transgene transcription at the whole plant level. Subsequently we tested conditions which would provoke the silencing again in these reactivated lines, since T-DNA insertions in these lines were demonstrably susceptible to spontaneous silencing. Such an opportunity to have a test for disqualification of silencing-susceptible lines in early stages of the selection process would be very tempting.

We treated potato plants (*Solanum tuberosum* L.; for more details read Chapter 3.1) with spontaneously silenced transgenes encoding the green fluorescent protein (GFP) and the neomycin phosphotransferase (nptII) by a demethylation drug, AzaC. Reactivation of transgene expression was assessed using quantitative real-time PCR (RT-qPCR) and methylation analysis. Plants with reactivated transgene expression were then tested in stress conditions that were hypothesized to potentially trigger re-silencing.

We determined 10µM AzaC concentration as an optimal for *S. tuberosum* treatment from experiments with apical cuttings, where 40µM concentration inhibited the growth, whereas 5µM was fully comparable with untreated control. The AzaC half-life was estimated to be approximately 2 days, because the inhibition effect of 40µM AzaC stored for 4 days in cultivation room correlated visually with cuttings treated by freshly added 10µM AzaC (Attachments 10.1.). GFP reactivation was no visible in shoot cuttings after AzaC treatment, possibly owing to limited transport efficiency of this drug. Therefore, we used *de novo* regeneration technique, where new shoot arises from a single cell of *calli*, which was in a contact with AzaC supplemented media. Selective media was also enriched with kanamycine for better selection of reactivated plants.

Combination of the selection marker, kanamycine (KAN), and AzaC caused stress and browning of the explants and inhibited the *calli* growth. Thus, we treated *calli* for four days by AzaC, separately from KAN treatment, which came after (Attachments 10.1.). This approach led to regeneration of several KAN resistant plants. Some plants derived from a multicopy line R17 also exhibited reactivated GFP expression, but only resistant plants with temporary GFP expression were derived from a single-copy line R28 (see Attachments 10.2.). Data were confirmed by the RT-qPCR analysis (Attachments 10.1.). To see whether reactivation of silenced transgene really correlated with promoter demethylation, methylation level of *Pnos* promoter and adjacent 5´part of *nptII* gene was determined in a single-copy line R28 and its reactivated "daughter" line R28A. Indeed, highly hypomethylated pattern of *Pnos* was observed in the reactivated line R28A with also hypomethylated *nptII* region (Attachments 10.1.).

Findings in this study indicate the possibility of using *de novo* regeneration method for early negative selection of transgenic *S. tuberosum* plants with tendency to silencing. Further analysis needs to be done to check applicability in other plant systems.

In conclusion, the most important findings of this work are:

- Transient 10μM AzaC treatment of *S. tuberosum* leaf segments during *de novo* regeneration gave rise to plants with restored expression of previously silenced transgenes at the whole plant level.
- Kanamycine and GFP expression was rescued in some regenerated plants and confirmed by RT-qPCR analysis and fluorescent microscopes.
- The methylation of *nptII* protein coding sequence and *Pnos* promoter region was significantly lower in plants regenerated after AzaC treatment.
- *De novo* regeneration of *S. tuberosum* plants from leaf segments could trigger transgene silencing and thus this procedure might be used to test susceptibility of transgenic plants to spontaneous silencing.

4.1.1. Statement of contribution

I, PhD candidate, hereby declare that I prepared reactivated lines, tested the factors affecting re-silencing and performed RT-qPCR analysis. Together with other authors I carried out the methylation analysis, microscopic evaluation, analyzed the data, summarized the results and wrote the manuscript and contributed to the revisions.

4.2. Publication 2 – (Čermák et al. submitted)

Unexpected variations in posttranscriptional gene silencing induced by differentially produced dsRNAs in tobacco cells

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Original research article, *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, (IF_{2018} = 4.599), submitted

While in first publication we studied the possibility of restoration transgene expression after its silencing, second publication was trying to respond to a question how can influence the origin of key RNAi regulator, sRNA, the development of PTGS. We selected the BY-2 as a model system, because it performs unique homogenously responding material with high proliferation rate, convenient for detail analysis by molecular techniques at single cell level. We aroused a *GFP* reporter gene silencing using XVE inducible system (Chapter 3.7.; Zuo et al., 2000) with the combination of three different silencers, in order to investigate its sensitivity, strength, time course, methylation rate or even sRNAs population.

We created three transgenic BY-2 lines by the transformation of different silencers into the line with stable GFP expression, namely AS (*GFP* in antisense orientation), IR (inverted repeat of *GFP*) and UT (unterminated *GFP*), all under the estradiol inducible promoter (Attachments 10.3.). Only lines with enough, high and homogenous GFP fluorescence were used in hundreds of calli for population analysis or in three suspension lines per variant for analysis in detail. GFP fluorescence of calli was assessed after estradiol application by G:BOX measuring efficiency, strength and speed of silencing. For detail analysis in suspensions, we determined at first the inducibility of XVE system by treatment of cells with estradiol in dilution series. After that we treated lines in suspensions with 2μM estradiol for 14 days and for other 21 days without estradiol, pinpointing by precise sampling GFP fluorescence at protein level (flow-cytometry) and transcript level (qRT-PCR), DNA methylation state (bisulfite conversion and McrBC assay) and sRNAs analysis (BGISEQ-500 sequencing).

We discovered from the population study that IR variant was fastest and strongest in silencing. AS variant was lower and UT at the same level as control (Attachments 10.3.). Strikingly, if construct of UT silencer with the start codon was transformed into the wt BY-2 cells, around 50 % of lines showed some GFP fluorescence (Attachments 10.4.). We also found out that some IR lines were reacting on even 500x lower estradiol concentration than generally used 2μM.

At the same time, cells were at the edge of the response to induction, because these low concentrations showed non-homogenous population of two states – active and silenced (Attachments 10.3., 10.4.). Time course of 14 days of estradiol application and 21 days without demonstrated that IR lines reacted homogenously, very quickly and returned after dropping off on the same level as before (transcription analysis of *GFP*; Attachments 10.3.). Simultaneously, high production of sRNAs during induction and declining after removal of ESTR (Attachments 10.3.) was accompanied by the massive target *GFP* methylation (Attachments 10.3.). AS lines were slightly slower compared to IR lines, but returned also on the same level as before (Attachments 10.3.). Level of sRNAs in AS lines was lower than IR and showed interesting shifted ratio in behalf of reverse strand (Attachments 10.3.), but there was no DNA methylation of target *GFP* during whole experiment (Attachments 10.3.). Reaction of UT lines after ESTR treatment was the slowest and the most heterogeneous. Nevertheless, after removal of the inducer many cells were still able to keep *GFP* silencing state (Attachments 10.3., 10.4.), which was confirmed by persisting level of sRNAs (Attachments 10.3.). UT lines also showed some DNA methylation, even established before induction (Attachments 10.3., 10.4.).

Ultimately, the most important findings of this work are:

- We established the experimental system based on transgenic BY-2 lines enabling the effective induction of silencing introduced transgene.
- Some lines from IR variant of *GFP* were possibly inducing silencing at 500 x lower ESTR concentration then usually used and exhibited specific binary silencing in cells.
- IR line further showed fastest and deepest silencing of GFP accompanying with the highest level of sRNAs and target DNA methylation.
- The line with AS of *GFP* was lower in response to silencing induction but demonstrated medium level of sRNAs with shifted ratio in favor of Rev strand and no target methylation.
- Some unterminated constructs can be translated to a functional protein.

4.2.1. Statement of contribution

I, PhD candidate, hereby declare that I participated together with other authors in the methylation, the RT-qPCR and the flow-cytometry analysis. I also contributed to the writing of the manuscript and approved the final version and revisions.

4.3. Publication 3 – Přibylová et al., 2019, published

Detailed insight into the dynamics of the initial phases of *de novo* RNA-directed DNA methylation in plant cells

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Original research article, *Epigenetics & Chromatin* 12:1 (IF_{2018} = 4.185)

DNA methylation of promoter cytosines gives rise to inactivation of expression following gene. In previous publications we focused on the reactivation of gene expression in *S. tuberosum* plants using demethylation drug and on the study of PTGS development when provoked by sRNAs of different origin. While in this work we wanted to deliberately elicit TGS in transgenic tobacco BY-2 cell line, in order to investigate not well-known initial phases of RdDM.

Three independent lines of BY-2 were prepared by the super-transformation of "mother line" expressing steadily GFP under *CaMV 35S* promoter (*P35S*). This second independent transformation event introduced into the "mother line" an inverted repeat of a part *P35S* under estradiol inducible promoter (Attachments 10.5.). Exponentially cultivated BY-2 lines were then either treated or not with estradiol to produce siRNAs against *P35S* in front of downstream *GFP*. For ten days we harvested cells in selected timepoints and analyzed the data for assessment of the silencer and target *GFP* level by the RT-qPCR, GFP fluorescence by the flow-cytometry, *P35S* methylation, and the existence of *P35S*-derived siRNAs by BGISEQ-500 sequencing.

GFP fluorescence of all three BY-2 lines, measured by the flow-cytometry in isolated protoplasts, was declining until loss in 10 days (Attachments 10.5.). Line 8 and 19 were chosen for deeper analysis because their response to estradiol was highly homogenous (Attachments 10.6.). Decrease of *GFP* transcript assessed by the RT-qPCR reached its minimum in 2 days while the transcription of hairpin quickly increased within the first 3 h, reaching maximum in 2 days (Attachments 10.5.). Difference between observed GFP fluorescence and absenting *GFP* transcript is given by the protein stability. We analyzed also the methylation state of not only the *P35S* target sequence (379 nt), but also in adjacent regions (104 nt up- and 82 nt down-stream). The target region was highly methylated in almost 80 % of C positions in two days after induction (Attachments 10.5.), whereas adjacent regions were methylated just slightly (Attachments 10.6.). There were observed also slightly different patterns in comparison with symmetric and asymmetric methylations (CHH declined after two days maximum, whereas CG and CHG gradually increased and got at maxima in 3 days; Attachments 10.5.). Analysis of siRNAs fitting

to both T-DNAs (comprising inducer and target part) showed some small differences between lines. Whereas siRNAs from line 8 aimed principally the target *P35S* region in both the inductor inverted repeat and target promoter, siRNAs from line 19 displayed besides same pattern also relevant number of siRNAs from hygromycin phosphotransferase (*hpt*) expression cassette (Attachments 10.6.). Surprisingly, low level of siRNAs against *P35S* were also found even in untreated cells. When we looked closer to the siRNA seq, the most siRNAs sitting along the target region were 21 nt and 22 nt in length; Attachments 10.5 and 10.6.). The coverage of the target sequence by siRNAs was not homogenous – there were found strand and line specific "peaks" with bigger siRNA density indicating higher siRNA stability or production (Attachments 10.5.).

Main findings of this work are:

- GFP fluorescence of all 3 lines was decreasing until its loss in 10 days, while the level of *GFP* transcript decreased in 2 days after induction in selected lines.
- The methylation of target sequence in *P35S* promoter came very fast, promptly after occurrence of relevant siRNAs, it reached its maximum in 2 days (in CHH) or escalated for 10 days (in CG and CHG).
- Formerly presented methyl-cytosines in target locus did not affect *de novo* methylation or its accuracy.
- The presence of RNA hairpin led to the high production of specific 21- and 22 nt siRNAs which covered not only *P35S* of target or inducer, but also *hpt* cassette.
- The coverage of target region showed some specific pattern of siRNAs in quantity.

4.3.1. Statement of contribution

I, PhD candidate, hereby declare that I carried out together with other authors the methylation, the RT-qPCR and the flow-cytometry analysis. I read and approved the final manuscript and revisions.

4.4. Unpublished data – Tyč et Fischer

Can plant cells import sRNAs from apoplast?

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Original research, presented at: 13th Student Days of Experimental Plant Biology, Czech Society of Experimental Plant Biology (CSEPB), 2015 September 7-8, Brno, Czech Republic. L1-4

The most important achieved data are included in the PhD thesis. However, the main goal to reveal the transport of sRNAs in plants with a help of special transporter proteins was not accomplished.

Investigation of little-known phenomenon of non-symplastic movement of a silencing signal was highly motivated by the observation made few years ago in the study of GFP-expressing tobacco BY-2 cell lines in our laboratory (Nocarová and Fischer, unpublished). Dr. Nocarová observed during her study focused on cloning of BY-2 cells (Nocarova and Fischer, 2009), that some GFP expressing lines silenced the GFP expression when mixed together with a line that had spontaneously silenced GFP expression. This effect could be caused either by the differences in proliferation rate of both lines – the silenced line could simply overgrowth the non-silenced one, or by spontaneous initiation of silencing in the line that until then had actively expressed GFP. Nonetheless, the silencing could also be hypothetically induced by the movement of a specific or a general silencing signal between the cells of the silenced and non-silenced line.

In very same year Slotkin et al. (2009) published a breakthrough article about the possible non-symplastic movement of TE-derived sRNAs in *A. thaliana* pollen (for more details read Chapters 1.4. and 1.5.). In plants, the silencing signal, in the form of sRNAs moves through PD, but the existence of another route for the sRNA transport between symplasticaly isolated cells can be expected. We checked the situation in animals, because the RNAi apparatus is conserved in many aspects in various Eukaryotes and cells in animal tissue have no interconnections like PD in plants. Indeed, in the near past SID-1 protein was discovered as a transmembrane transporter of dsRNAs, precursors of sRNAs, in nematodes (read more in Chapter 1.6.; Winston et al., 2002; Feinberg and Hunter, 2003).

4.4.1. Plant *SAG18* as a candidate homolog gene to *SID-1*

SID-1 protein sequence (NM_071971.7; NCBI GenBank; The *C. elegans* Sequencing Consortium, 1998) was used to find possible plant homologs in *A. thaliana* using tBLASTn. We obtained *Senescence Associated Gene 18* (*SAG18*; NM_105788) with unknown function (Miller et al., 1999; see Fig 4.4.1a for protein alignment). Another tBLASTn search was done, using SID-1 protein homolog of *Dictyostelium discoideum* (XM_001732989.1; NCBI GenBank; Eichinger et al., 2005) that belongs to a different phylogenetic branch Amoebozoa, with the same hit in SAG18. The reverse searches with SAG18 as a query returned back to SID-1 proteins as the closest homologs in animals and *D. discoideum*.

Also, other possible homologues of SAG18 in *A. thaliana* were checked using BLASTp (PSI-BLAST algorithm), that found putative alkaline phytoceramidase (aPHC; NP_001190292.1; Tabata et al., 2000; see all 3-protein alignment in Chapter 9.1.). To further compare the SID-1 and its putative homologs in plants, secondary structures of *C. elegans* SID-1 and *A. thaliana* SAG18 were calculated by PROTTER (Omasits et al., 2014) and Phobius webtool (combined transmembrane topology and signal peptide predictor; Figure 4.4.1b and 4.4.1c). Both proteins were predicted to have multiple transmembrane helices, but their number was higher in SID-1, which also differed in having a great extracellular domain (Fig. 4.4.1a-c and Figure 1.6a from Li et al., 2015). This part also includes some key residues for protein function (see alignment in Chapter 9.1.; Li et al., 2015). Other important residues for *C. elegans* SID-1 protein function are in homological regions, from which Ser-536 is highly conservative also in SAG18 or aPHC homological positions (see alignments in Chapters 9.1. and 9.2.; Li et al., 2015).

Comparison of selected green plants (Viridiplantae) SAG18 homologs showed high sequence conservation (see Figure 9.1. and phylogenetic tree in 9.3.). Therefore, we checked also the expression profile in *A. thaliana* by the Genevestigator database. It was shown that SAG18 is highly expressed in many tissues with the first positions occupied by sperm cells and stomata guard cells that are symplastically isolated and in the hairy root cells, which are at the interface with the environment and whose cell wall is permeable for water solutes (Figure 4.4.1d).

Although the predicted structures of SAG18 and SID-1 differ, it could be possible that the function done by SID-1 was split in the plant evolution into two separate proteins, one of which is SAG18. So, we decided to study SAG18 and aPHC as proteins that could be responsible for the SID-1 function, transmembrane transport of RNA, in *A. thaliana*. As a model for the investigation of SAG18 role we used tobacco BY-2 cell line and for the whole plant study, we chose *A. thaliana* for its available wide collection of mutant lines.

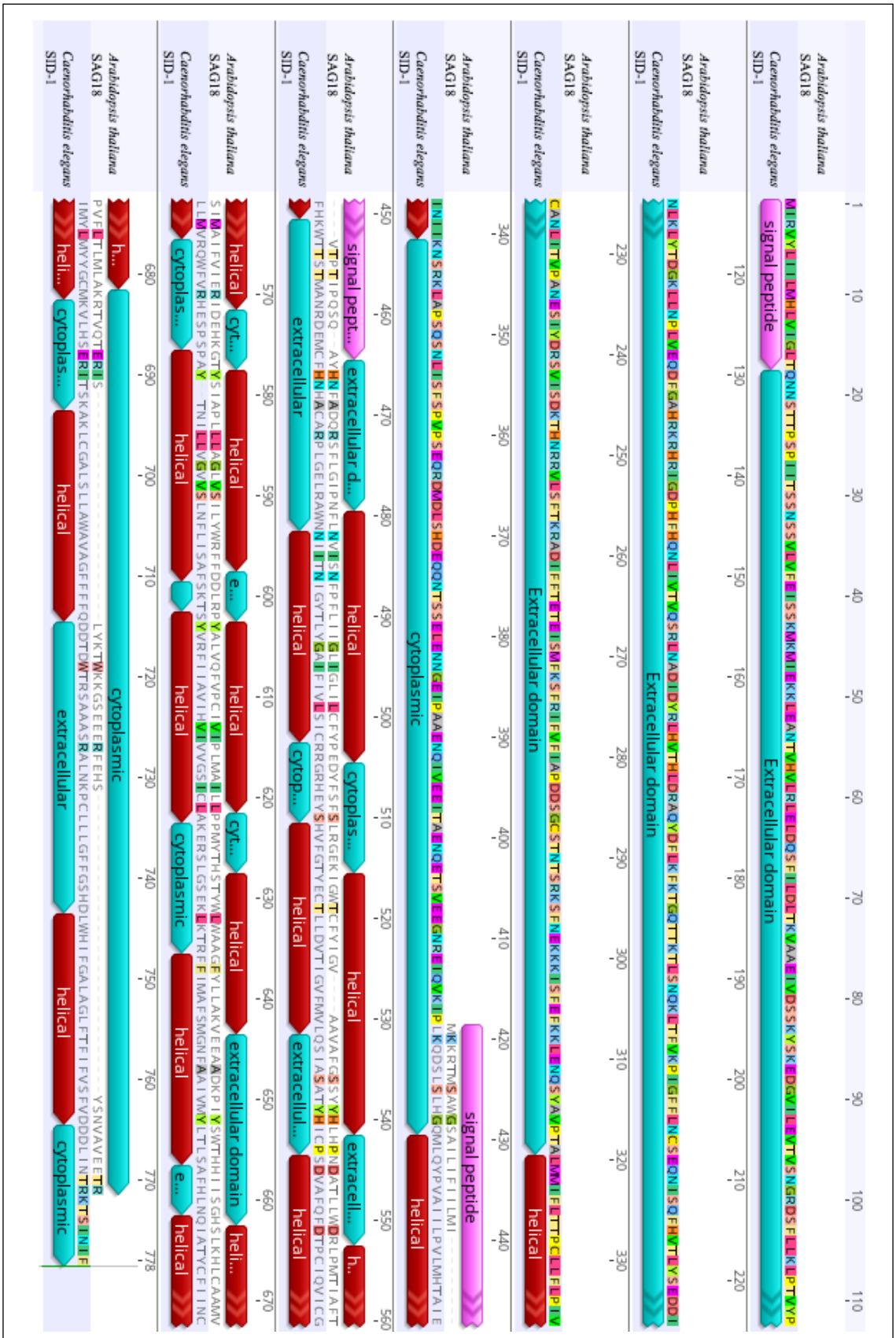


Fig. 4.4.1a Protein alignment of *Arabidopsis thaliana* SAG18 and *Caenorhabditis elegans* SID-1 with highlighted protein topology (calculated by Phobius for SAG18 and for SID-1 from Li et al., 2015).

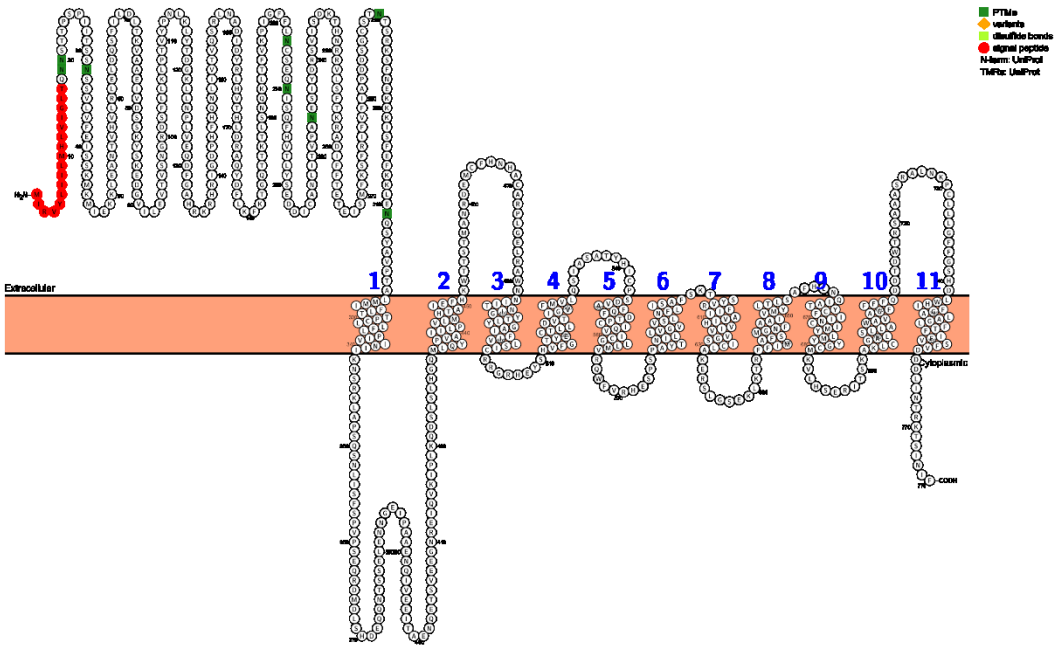


Fig. 4.4.1b Model of *Caenorhabditis elegans* SID-1 protein secondary structure (calculated by the Protter from the UniProt data Q9GZC8).

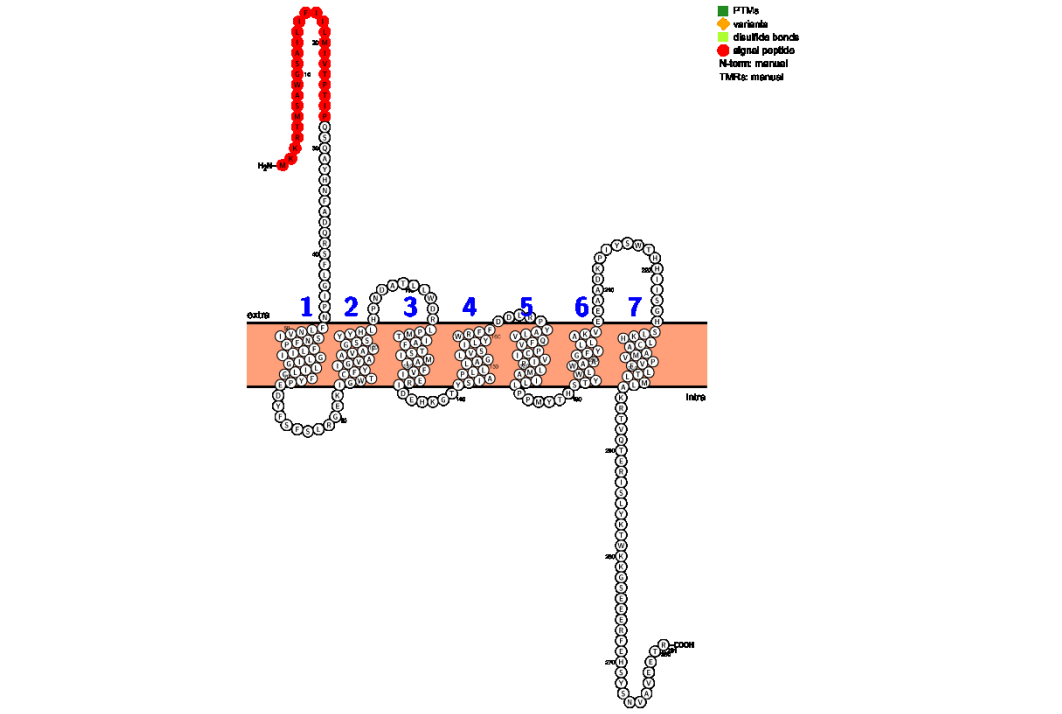


Fig. 4.4.1c Model of *Arabidopsis thaliana* SAG18 protein secondary structure (calculated by the Protter from the Phobius data Q9C989).

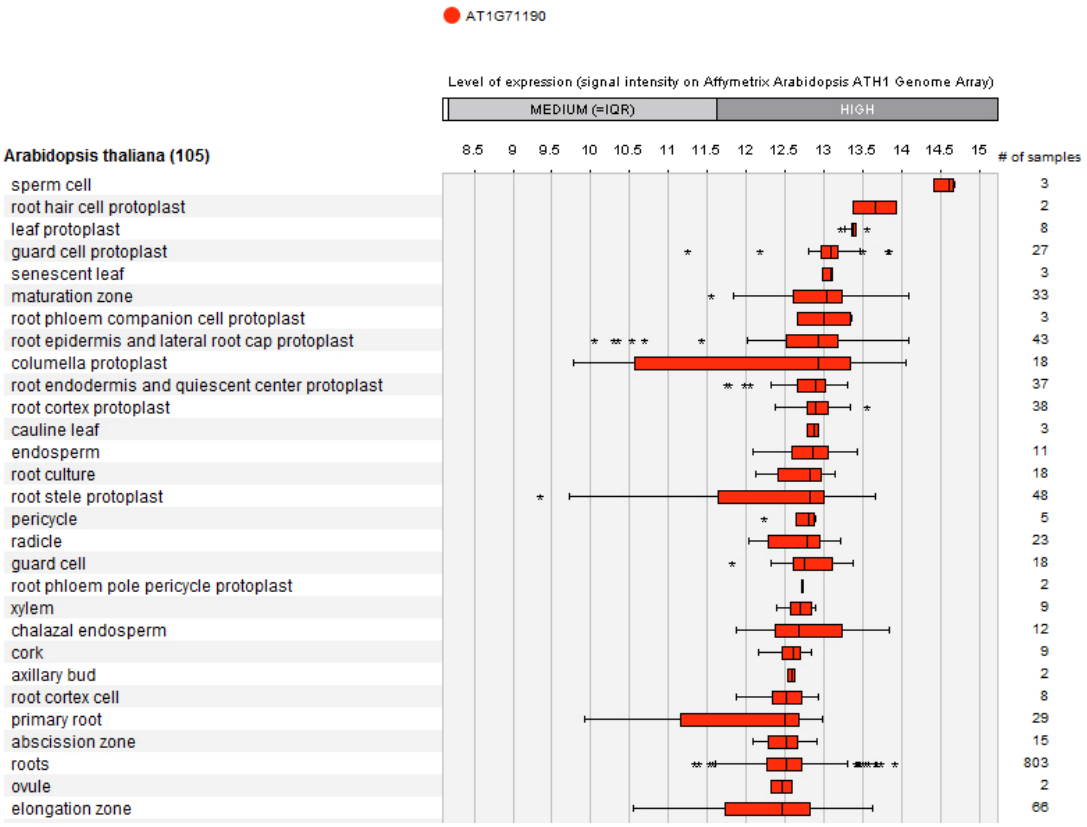


Fig. 4.4.1d Level of *Arabidopsis thaliana* SAG18 expression intensity on Affymetrix genome array (Genevestigator).

4.4.2. *Arabidopsis thaliana* double mutant preparation and phenotyping

We ordered seeds of *A. thaliana* (Col-0) T-DNA insertion mutants from the SALK collection (see more in Table 4.4.2a) and designed primers for genotyping (see more in Table 9.4) to select homozygous mutants in F2 generation. Neither *sag18* nor *aphc* showed any obvious phenotypic alterations (see Figure 4.4.2a). We cross them to obtain in F2 generation double mutants, *sag18 aphc*, which were again with no visible change of phenotype (see Figure 4.4.2a). We grew these mutants for additional three inbred generations to let some possible changes in phenotype manifest, with the idea that SAG18 could be involved in TE inactivation in pollen grain, thus the phenotype changes caused by TE reactivation could develop through generations (considering Mirouze et al., 2009; Slotkin et al., 2009). But no visible changes in the phenotype of any mutant appeared (see Figure 4.4.2a).

Tab. 4.4.2a *Arabidopsis thaliana* SALK mutant lines used in this study.

polymorphism	locus	gene	polymorphism site	NASC stock	phenotype
SALK_022062	AT1G71190	*SAG18*	exon	N522062	non available
SALK_003875	AT5G11870	*aPHC*	exon	N503875	non available

Since *SAG18* and *aPHC* are highly expressed in various tissues (for *SAG18* see Figure 4.4.1.d, for *aPHC* data are not shown) and there is no other homologue present in the *A. thaliana* genome, we tried to find any phenotype alteration in the mutant plants that would help to reveal the function of these proteins, regardless of whether in sRNA transport or in another processes.

As the *SAG18* gene is the most expressed in sperm cells, we first tested the efficiency of mutant alleles transmission to the next generation progeny, but no statistically significant difference between the theoretical and the experimentally determined ratio of the respective genotypes was observed in the progeny of self-fertilization of heterozygous *SAG18 sag18 aPHC aphc* plant (see Tables 4.4.2b-c).

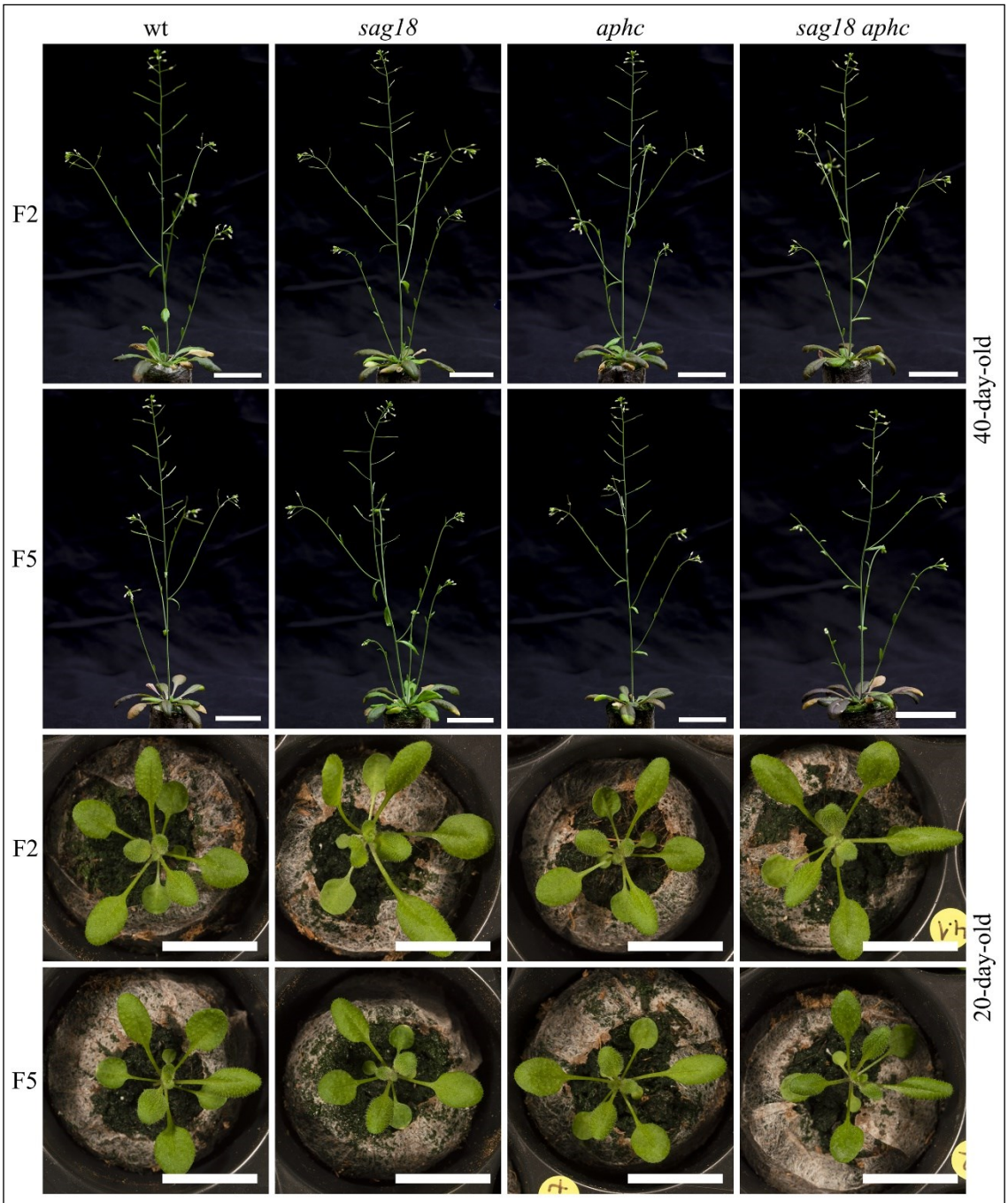


Fig. 4.4.2a F2 and F5 generations after self-fertilization of *A. thaliana* wt, *sag18*, *aphc* and *sag18 aphc* double mutants, grown in 16 h long-day (LD) photoperiod in Jiffy pelets with no visible differences in phenotype. Scale bar for 20-day-old seedlings is 2 cm, while for 40-day-old plants is 4 cm.

Tab. 4.4.2b Phenotypic ratio in F2 generation after self-fertilization (28 plants) of heterozygous *Arabidopsis thaliana* mutant plants for both genes **together**.

genotype	**AABB**	**AABb**	**AaBB**	**AaBb**	**AAbb**	**Aabb**	**aaBb**	**aaBB**	**aabb**
# theoretical	1/16	2/16	2/16	4/16	1/16	2/16	2/16	1/16	1/16
% theoretical	6.25	12.5	12.5	25	6.25	12.5	12.5	6.25	6.25
# real	**1/28**	**5/28**	**3/28**	**5/28**	**1/28**	**5/28**	**6/28**	**2/28**	**0/28**
% real	**3.57**	**17.86**	**10.71**	**17.86**	**3.57**	**17.86**	**21.43**	**7.14**	**0**

Pearson's Chi-squared test, p-value = 0.1006

Tab. 4.4.2c Phenotypic ratio in F2 generation after self-fertilization (28 plants) of heterozygous *Arabidopsis thaliana* mutant plants for each gene **separately**.

genotype	**AA**	**Aa**	**aa**	**genotype**	**BB**	**Bb**	**bb**
# theoretical	1/4	1/2	1/4	**# theoretical**	1/4	1/2	1/4
% theoretical	25	50	25	**% theoretical**	25	50	25
# real	**7/28**	**13/28**	**8/28**	**# real**	**6/28**	**16/28**	**6/28**
% real	**25**	**46.43**	**28.57**	**% real**	**21.43**	**57.14**	**21.43**

Pearson's Chi-squared test, p-value = 0.9249

We also checked other important parameters, which could be affected by the mutation in *SAG18* or *aPHC* genes. We focused on the measurement of parameters related to the tissues where both genes are highly expressed (see Figure 4.4.1d) – the yield and the stem and hairy roots parameters. We compared the wt and the double mutant (30 plant per genotype), because the most severe differences may be expected due to mutation of both genes. When stems were fully developed, they were assessed per each plant. Immediately after senescence, during which SAG18 expression increases, all desiccated plants were harvested and measured separately for the shoot and the total seed weight. Index ratio was determined as the ratio weights of harvested seeds to the total shoot for each plant (for more details see methods in Chapter 3.9.). But again, no significant differences were found out between the wt and the double mutant plants (see Figure 4.4.2b – charts **a**, **b**, **c** and **d**. Hairy root assessment was done in three biological replicates with 12-15 plants of each variant (wt, *sag18*, *aphc*, *sag18 aphc*; see Figure 4.4.2c) as described in methods (Chapter 3.10.). No differences were seen for the number of hairy roots per plant or for their length between any variants (Figure 4.4.2b – charts **e** and **f**).

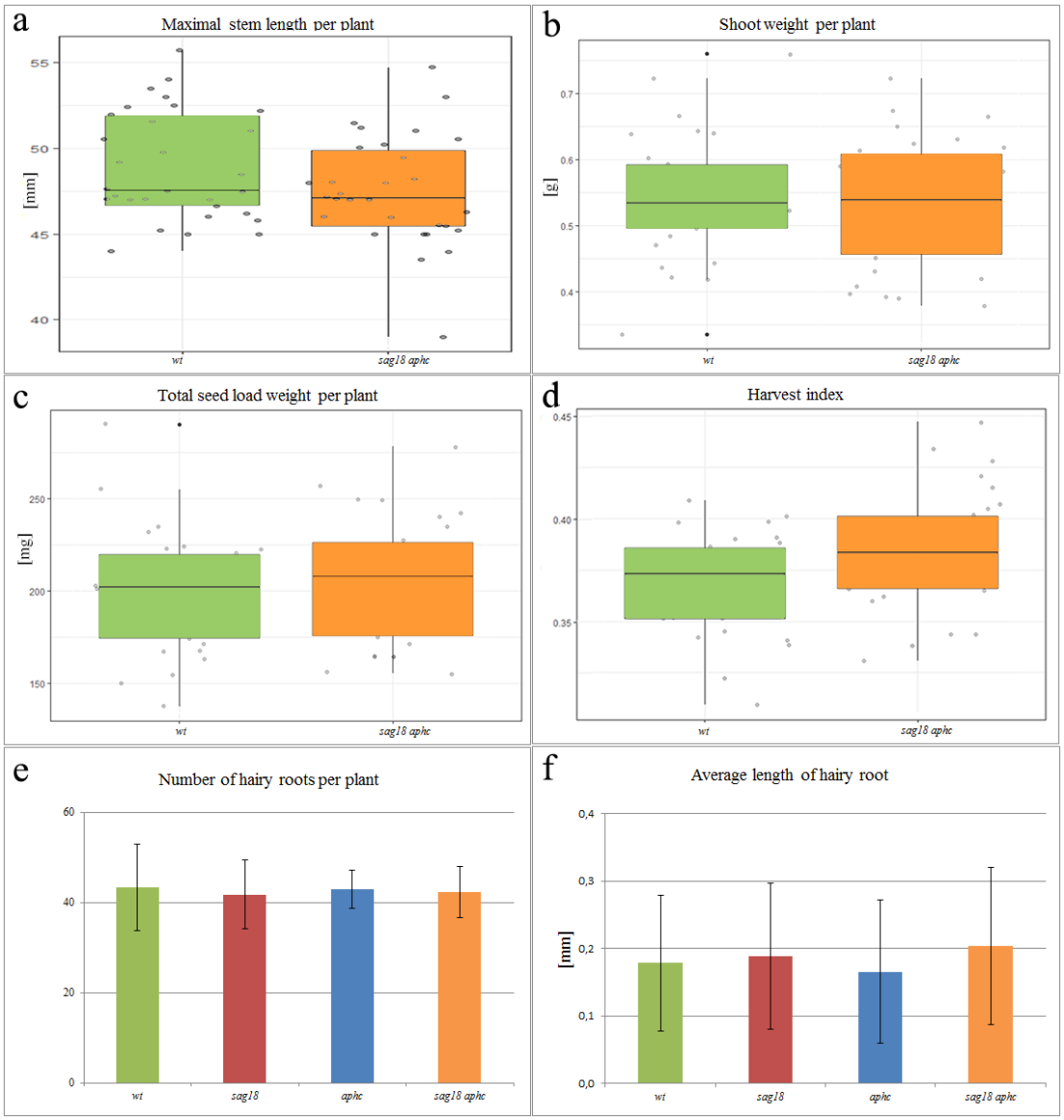


Fig. 4.4.2b Phenotyping of *Arabidopsis thaliana* wt, *sag18*, *aphc* and *sag18 aphc* double mutants grown in 16 h long-day (LD) photoperiod on the soil for (**a**, **b**, **c** and **d**) or on the MS/5 medium for (**e**, **f**). Stem length in (**a**) was calculated as a maximal length per each plant. (**b**) and (**c**) were measured when plants were desiccated. Harvest index in (**d**) was calculated as the ratio of harvested seeds to total shoot for each plant. And hairy roots were measured from pictures of 4-6 days old seedlings rooting *in vitro* on vertical agar plates using NIS-elements software. Error bars indicate the SD of 12-15 plants. Statistically there were no significant differences between wt and mutants for all measurements using linear models.

Fig. 4.4.2c Hairy root analysis of *Arabidopsis thaliana* wt, *sag18*, *aphc* and *sag18 aphc* double mutants grown in 16 h long-day (LD) photoperiod on MS/5. Scale bar 4 mm. The white arrows define measured part of a root under hypocotyl, an enlargement on the left is showing hairy roots in detail.

RNA movement (thus possible *SAG18* and *aPHC* involvement) is very important also in trans-kingdom host-parasite interactions (read more in Chapter 1.5.; Liu and Chen, 2018). Considering no phenotype changes in double mutant plants (for the putative transporters) we decided to check if they could not be involved in response to pathogen infection. *Botrytis cinerea* was selected as a suitable pathogen, in which mutations in proteins important for sRNAs transport lead to higher pathogenicity (Cai et al., 2018). We analyzed the size of lesions developed after *B. cinerea* in *A. thaliana* plants (5 plants per genotype – wt, *sag18, aphc, sag18 aphc*; for more details read Chapter 3.11.). As in all previous comparisons, there were no statistically significant differences (see results in Figure 4.4.2d).

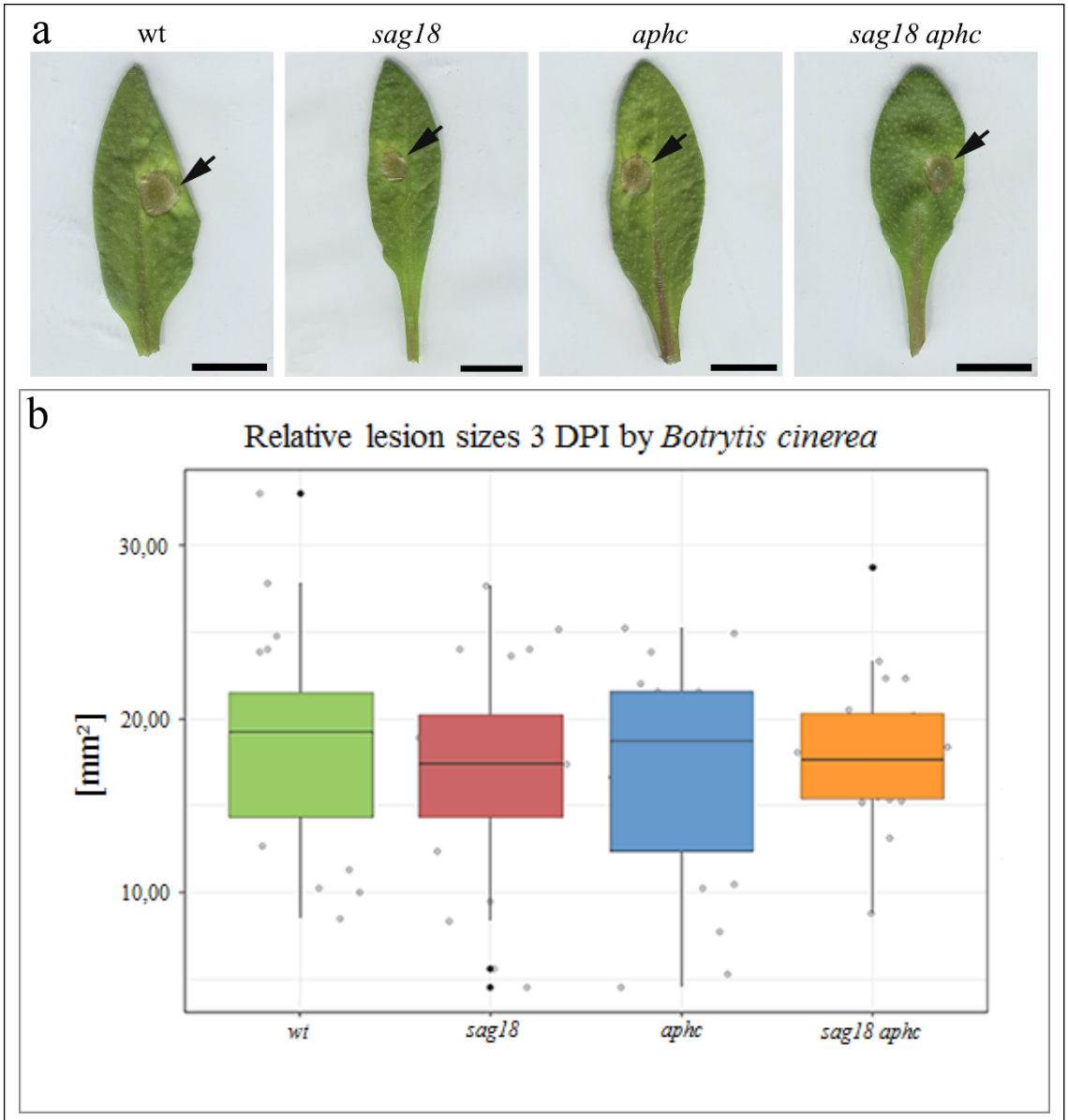


Fig. 4.4.2d Infection of *Arabidopsis thaliana* wt, *sag18*, *aphc* and *sag18 aphc* double mutants by *Botrytis cinerea*. (**a**) *A. thaliana* leaves after 3 DPI (days post inoculation) by fungi, scale bar 10 mm. (**b**) relative lesion sizes of *A. thaliana* leaves. Error bars indicate the SD of 20 leaves. No statistical difference was found between variants using linear model.

4.4.3. Guard cell analysis reveals possible differences

We also evaluated guard cell function, aware of the high expression rate of SAG18 (Fig. 4.4.1d) and aPHC, in order to find their possible alternative function. The hypothesis was, that the mutation in both genes may negatively affect guard cell function that would result in altered stomata conductance and thus also the rate of transpiration. We applied two methods to analyze stomatal function on three different genotypes (wt, *sag18*, *sag18 aphc*) – the photosynthesis measuring system TPS-1 and the gravimetric method (Chapter 3.12.). Both approaches showed interestingly the same trends of reduced speed of stomatal closing in the double mutants. Plants

with *sag18* and *sag18 aphc* mutations exhibited significantly higher water loss (see Figure 4.4.3 **c**) and concurrently higher stomatal conductance and transpiration rate measured by TPS-1 (see Figure 4.4.3 **a**, **b**). Deeper analysis using all possible genotypes in quantity larger set of plants and inclusion of other *A. thaliana* guard cell mutants should be done to verify the results.

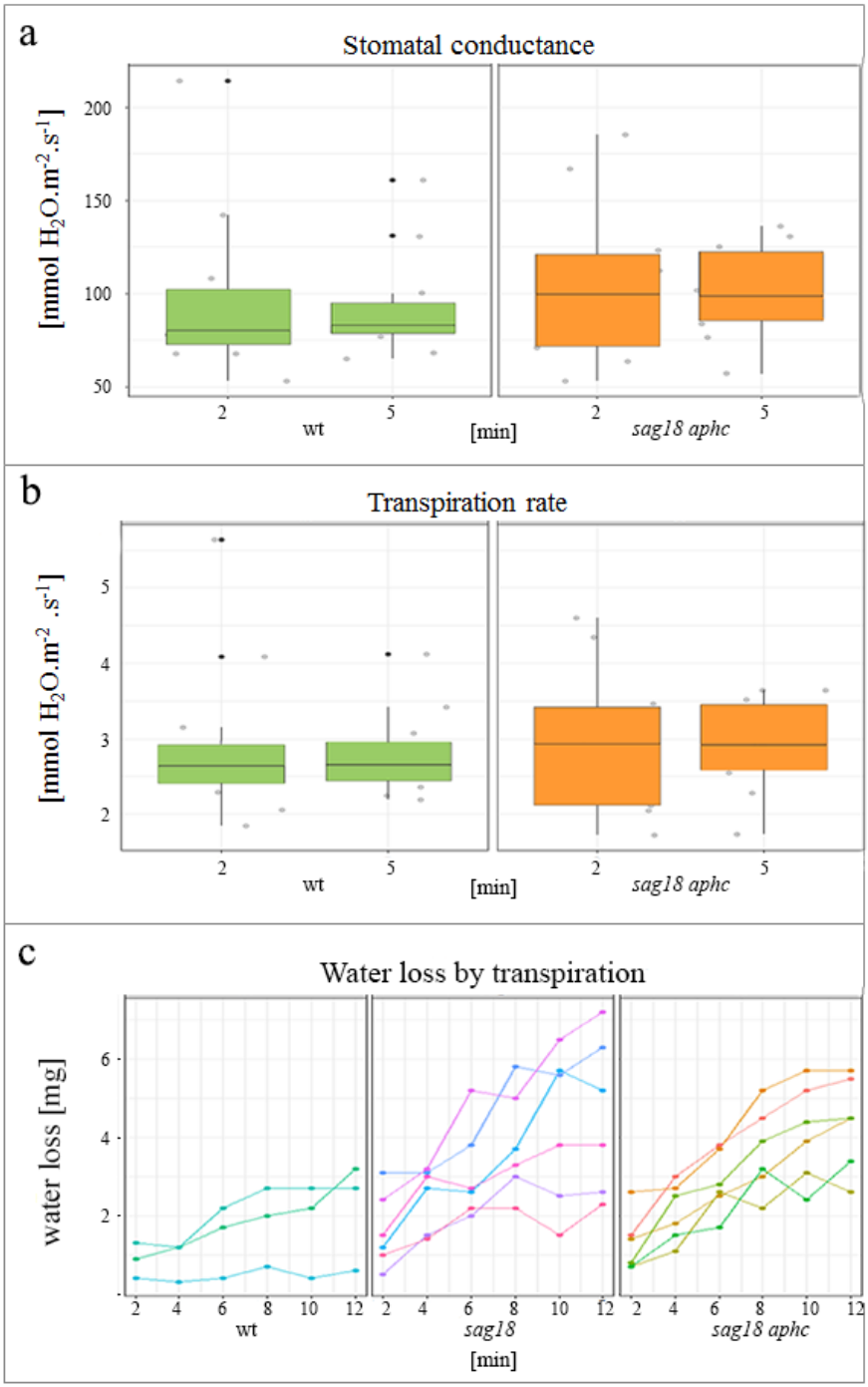


Fig. 4.4.3 Stomata guard cell analysis of *Arabidopsis thaliana* wt, *sag18*, and *sag18 aphc* double mutants measured for (**a**, **b**) by photosynthesis measuring system TPS-1 and for (**c**) using gravimetric method. (**a**, **b**) stomatal conductance and transpiration rate was measured every 2 and 5 minutes. Error bars indicate the SD of 11 leaves. (**c**) water loss by transpiration was evaluated every 2 minutes using gravimetric method together for 12 minutes. The lines represent 3-6 leaves. Statistics showed difference in pairs wt:*sag18 aphc* (p-value = 0,007) and wt:s*ag18* (p-value = 0,0027) using linear mixed-effects model with post-hoc Tukey's HSD test.

4.4.4. Investigation of sRNA movement between individual cells of BY-2 cell line

Tobacco BY-2 cell line represents an important plant model of mitotically dividing cells with few limits (absence of mutant lines and genome instability; Kovarik et al., 2012, not later confirmed by Srba et al., 2016), but also with many advantages (high proliferation rate, easy handling, easy transformation and selection, easy epigenetic marks investigation; Nagata et al., 1992; Srba et al., 2016; Přibylová et al., 2019; Čermák et al., submitted).

As mentioned above, we observed that the percentage of cells with silenced GFP expression increased in time in the mixture of BY-2 cells actively expressing GFP and cells that had the expression spontaneously silenced. To study this phenomenon, we established more controlled experimental system, which fulfilled following requirements – i) active expression of SAG18 homolog; ii) simple differentiation between cell populations that act as putative donors and acceptors of the silencing signal (sRNA); iii) efficient production of specific sRNAs in the donor cells; iv) possible overcome of mechanical barrier in sRNAs movement and; v) simple detection of sRNAs movement effects.

Weak transcription of tobacco homolog of SAG18 in BY-2 cell line was proved by semi-quantitative RT-PCR amplification of *SAG18* from cDNA using primer pairs 5´-CTGAGCAGAACTTGAGCTTC-3´ and 5´-TCCCTTCAACGTGATTCCTC-3´ (Fig. 4.4.4a).

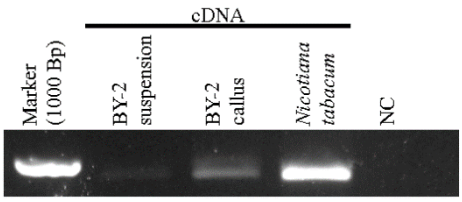


Fig. 4.4.4a PCR amplification of *SAG18* from cDNA of BY-2 suspension (faint band), BY-2 callus and *Nicotiana tabacum* (positive control). NC means negative control. 1.2% agarose gel in 0.5x TAE. In-gel staining by GelRed (Biotium, Inc.). Captured and processed by G:BOX (SynGene).

The first approach was to mix two BY-2 lines with different T-DNA background and the potential for sRNA movement observation. Binary vector with two independent fluorescent marker proteins was prepared by classical recombinant DNA techniques (GRED1; see Tab 3.1. and Fig. 3.1a-**c**) and transformed into BY-2 creating putative acceptor line. IR8C putative sRNAs donor line contained *P35S::*GFP and the cassette for *GFP* as inverted repeat under estradiol inducible promoter (see Tab 3.1; Chapter 3.7.; Fig. 3.1a-**b** and Čermák et al. submitted). The hypothesis was that sRNAs against *GFP* will be produced in response to the estradiol induction and they will move from the IR8C cells into the GRED1 cells, where they negatively

affect *GFP* expression. We expected that in such a mixture of BY-2 cells, we would see two types of cells – without any fluorescence (from IR8C, with GFP silenced by the internal sRNAs) and cells with red fluorescence of mCherry, but reduced GFP fluorescence (from GRED1, where the GFP was silenced by external sRNAs). However, GRED1 showed weak mCherry fluorescence. Thus GRED2 (Tab 3.1 and Fig. 3.1a-**d**) was created using *RFP* and subsequently GRED213 and GRED235 lines with two independent T-DNA insertions were selected and used in further analysis.

Line IR8C was at first tested for sufficient sRNAs production by the estradiol treatment (Chapter 3.7.). Fast reduction of GFP expression proved massive production of sRNAs against *GFP* in the treated line and its usability as a potential donor of sRNAs (see Figure 4.4.4b). This was also later confirmed in detail by flow-cytometry in Publication 2 (Chapter 4.2., Attachments 10.3. and 10.4., Čermák et al. submitted). In the pilot experiment, IR8C was induced and after 72 h mixed with GRED213 or GRED235 lines with no visible changes in GFP fluorescence after another 72 h (data not shown).

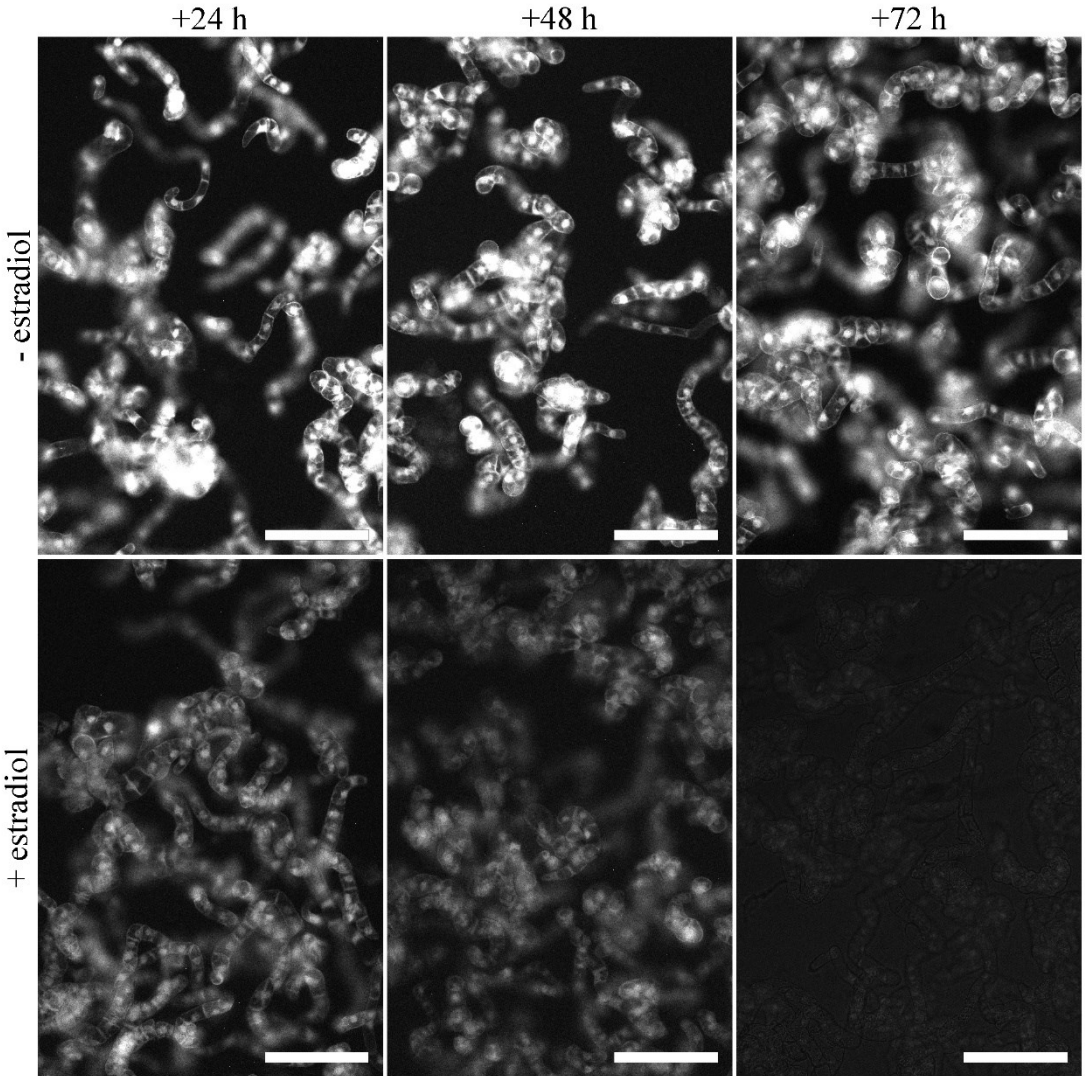


Fig. 4.4.4b Pictures of tobacco BY-2 cell line IR8C from fluorescent microscope showing a reduction in *GFP* expression during continual estradiol treatment. Processed by Adobe Photoshop CS6. Scale bar 200 μm.

4.4.4.1. Externally supplied sRNAs against *GFP* did not cause changes in GFP fluorescence of the acceptor line

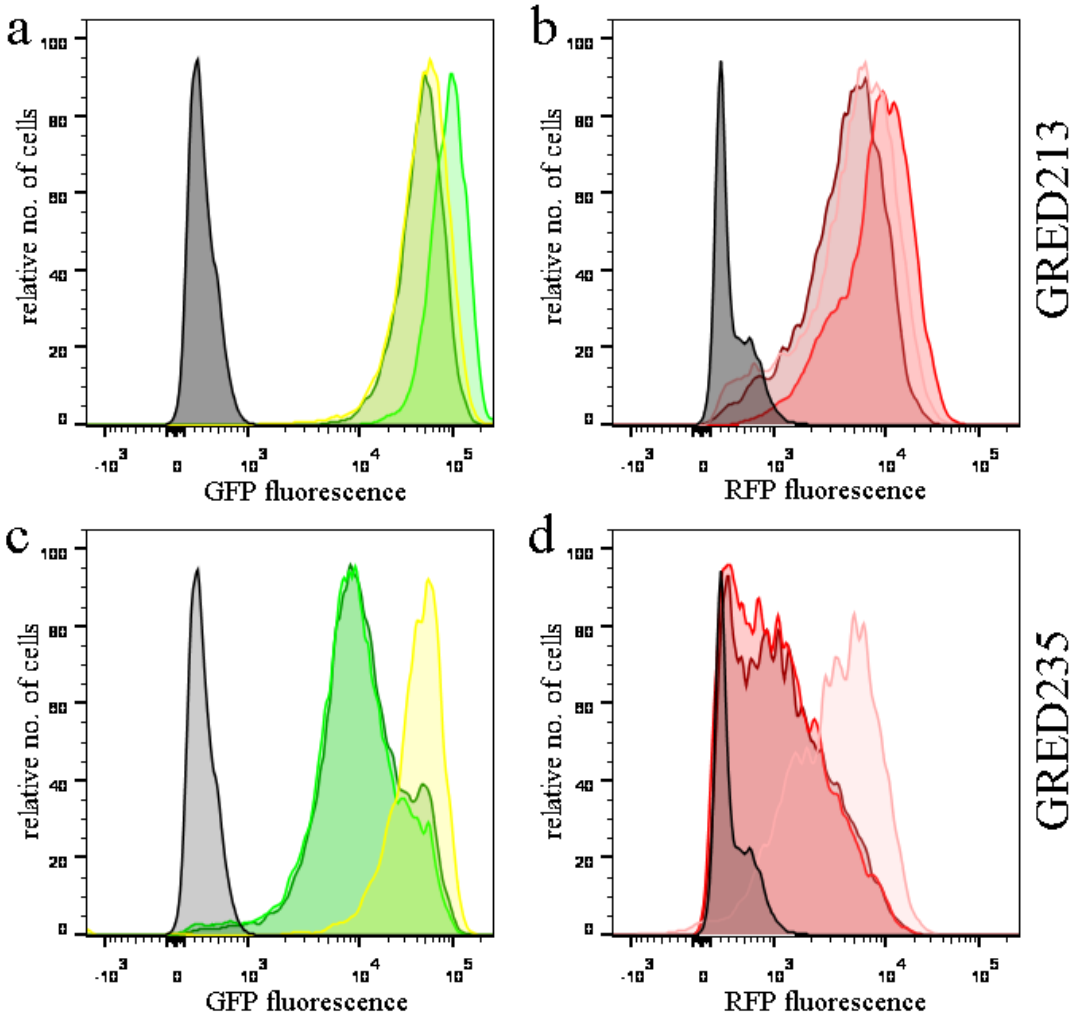
Considering that SID-1 (putative SAG18 homolog) was shown to be important for dsRNA uptake, but not for the export (Shih and Hunter, 2011), we decided to use frozen IR8C donor line. The application of liquid nitrogen on IR8C sample might improve the release of dRNAs after the destruction of cells during thawing.

Line IR8C was treated with estradiol or DMSO (control) and harvested after three days of induction (read more in Chapter 3.7; see Fig. 4.4.4b). Each frozen sample contained 0.5 ml of IR8C line presumably with or without sRNAs against *GFP*, depending on the estradiol treatment. Two acceptor lines (GRED213 and GRED235) were grown as suspensions under

conditions described above (Chapter 3.1.). When lines achieved homogeneity and exponential growth (sub-cultivation interval 3-4 days), both were treated with sterile frozen 0.5 ml of IR8C (with or without estradiol addition before freezing). After four days they were analyzed for GFP expression without any visible differences (Chapter 3.3; data not shown). Therefore, both lines were treated again with frozen IR8C, this time with 5 ml per variant. After three days there was no sign of fluorescence changes between variants, so another dose of frozen cells was added. Each line was overall treated with 11 ml of frozen IR8C cells per variant. Lines were kept in the exponential growth for another 14 days and then harvested and analyzed by flow-cytometry (Chapter 3.3.).

Strikingly GFP fluorescence was lower in GRED213 after the putative sRNA treatment (46% decrease compared to the line treated with estradiol-induced frozen IR8C; Fig. 4.4.4.1a-**a**). Nevertheless, trends in RFP fluorescence were almost the same (47% decrease after the treatment; Fig. 4.4.4.1a-**b**) although we expected that sRNAs from IR8C line would affect only the fluorescence of GFP. In the second analyzed line GRED235 fluorescence of both GFP and RFP declined about three times compared with untreated control. This decrease was practically equal in both IR8C-treated variants irrespective of their estradiol treatment (Fig. 4.4.4.1a-**c**, **d**).

The above described experiments did not indicate that our hypothesis that sRNAs can move between BY-2 cells or that sRNAs from the medium can be internalized by BY-2 cells to influence the GFP fluorescence. Nevertheless, presumed small decrease in GFP fluorescence after sRNAs treatment could be masked by newly synthesized GFP if the imported sRNAs are unable to induce production of secondary sRNAs in the recipient cell. Thus we considered to block the synthesis of new fluorescent proteins (which could hide a potential small decrease of GFP after sRNAs treatment) by adding cycloheximide (CHX; Imanishi et al., 1998). Simultaneously, we also tried to prevent any possible barrier (like cell wall) for sRNAs uptake on the recipient side, therefore we decided to treat freshly prepared protoplasts (Chapter 3.8.).



line	treatment	# count	# count live	% live	GFP mean	RFP mean
GRED213	none	30000	22640	76	54152	7057
GRED213	IR8C+0	7883	5185	66	93508	10783
GRED213	IR8C+ESTR	30000	5914	20	50213	5670
wt	none	30000	5922	20	329	393
GRED235	none	30000	3622	12	46410	4720
GRED235	IR8C+0	30000	16090	54	15165	1710
GRED235	IR8C+ESTR	30000	13345	45	17366	1859
wt	none	30000	5922	20	329	393

Fig. 4.4.4.1a Fluorescence of reporter proteins in protoplasts of tobacco BY-2 cell line after estradiol treatment measured by low-cytometry. (**a**) GFP and (**b**) RFP fluorescence of GRED213 line; (**c**) GFP and (**d**) RFP fluorescence of GRED235 line; the colours of curves in the histograms are equivalent to those in table of mean.

Protoplasts of GRED213 and GRED235 lines were treated with 100µM CHX according to Imanishi et al. (1998) to investigate if CHX could effectively block synthesis of new GFP. Fluorescence of GFP and RFP was measured for three days using flow-cytometry. Data showed decrease in fluorescence of GFP independently of treatment but strikingly increase in RFP fluorescence during time (Fig. 4.4.4.1b). This could be caused by differences in lifetime of both proteins, considering mostly dropping ratio of living cells during time because of stress from long time protoplasts cultivation (Tab. 4.4.4.1b). Difference in GFP between treated and untreated variants was expected in opposite way – CHX treatment was assumed to lead to decrease of GFP because of blocking new proteins synthesis. But we did not realize that CHX is also blocking a synthesis of proteasome proteins (McKeehan and Hardesty, 1969) thus the lifetime of proteins could be prolonged.

Tab. 4.4.4.1b Flow-cytometry data from GRED lines treatment with cycloheximide.

Line	**treatment**	**day**	**# count**	**# count live**	**% live**	**GFP mean**	**RFP mean**
GRED213	none	0	30000	14788	49	59290	4465
GRED213	CHX	1	30000	11188	37	46642	5754
GRED213	none	1	30000	10967	37	48620	5578
GRED213	CHX	2	30000	11100	37	47210	6531
GRED213	none	2	30000	3338	11	44625	6527
GRED213	CHX	3	31355	4146	13	40538	6932
GRED213	none	3	24130	6797	28	16365	7809
GRED235	none	0	30000	9448	32	24485	2746
GRED235	CHX	1	30000	10464	35	20970	3657
GRED235	none	1	30000	5323	18	20954	3323
GRED235	CHX	2	30000	3919	13	17335	3827
GRED235	none	2	30000	4269	14	11433	4684
GRED235	CHX	3	11660	4835	42	20213	4313
GRED235	none	3	3545	79	2	10622	4078

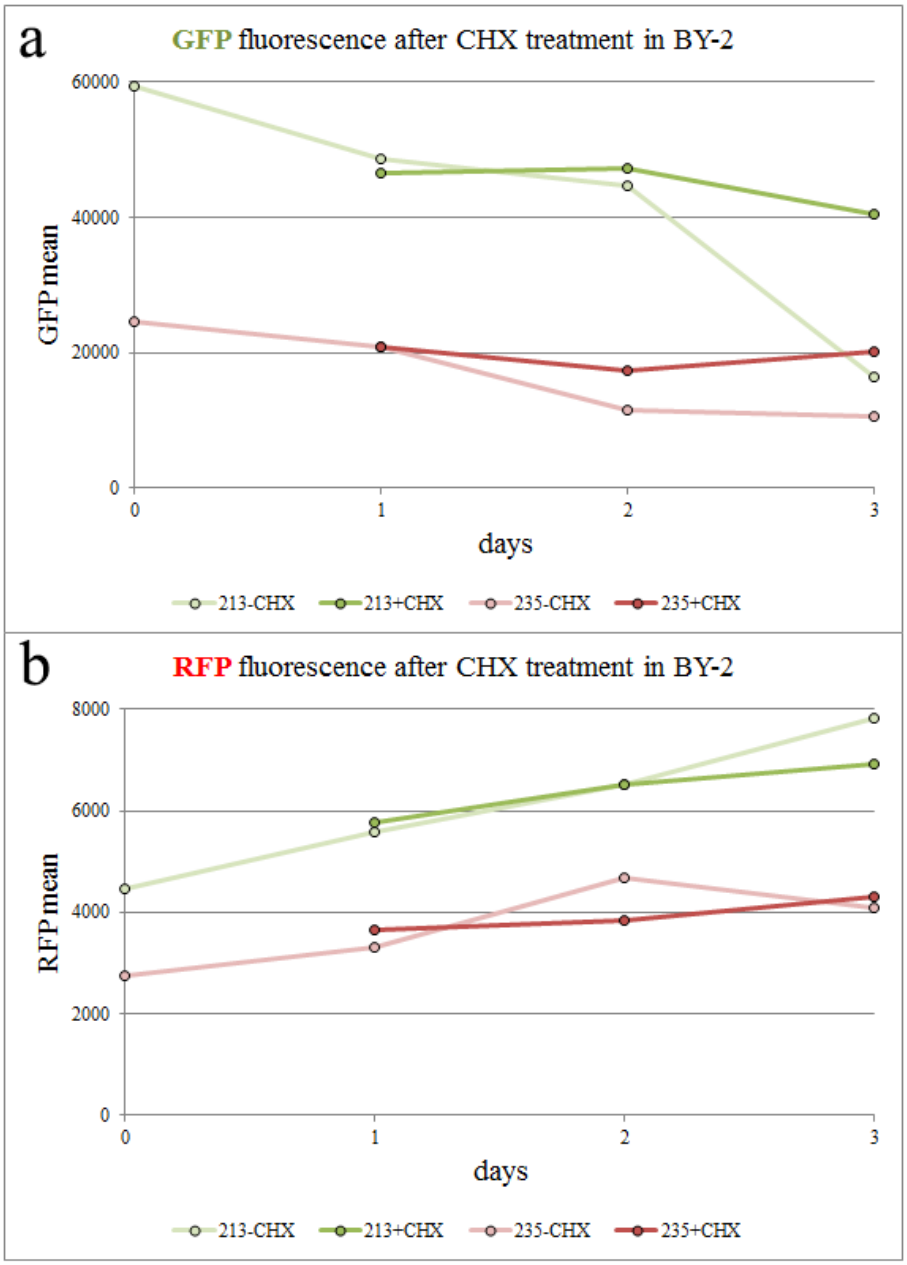


Fig. 4.4.4.1b Time course of GFP (**a**) and RFP (**b**) fluorescence in two GRED2 tobacco BY-2 cell lines after CHX treatment, measured in protoplasts by flow-cytometry.

4.4.4.2. Externally supplied sRNAs against *GFP* did not cause changes in *GFP* transcript level of the acceptor line

Since the CHX treatment did not help to prevent possible problematic monitoring of small decrease in GFP fluorescence after sRNAs treatment we decided to investigate the level of *GFP* rather at the transcript basis by RT-qPCR analysis. Concurrently we prepared also another transgenic BY-2 lines with overexpressed *SAG18* or even *SID-1* proteins to intensify the changes in GFP expression due to sRNAs movement. Furthermore, we applied the RiboLock RNase

inhibitor during sRNAs solution preparation to protect sRNAs before RNase degradation (Chapter 3.7.).

Transgenic BY-2 lines were prepared, increasing the level of – i) *SAG18* gene from *A. thaliana* or ii) already known transporter of sRNAs – *SID-1* from *Caenorhabditis elegans*, both these genes controlled by strong *CaMV 35S* promoter. Thus, GRED213 line was super-transformed either by *P35S::AtSAG18* or *P35S::CeSID-1*, formatting GRSA respectively GRCE lines (see Chapter 3.1., Fig. 3.1b-**a**, **b**, Tab. 3.1). The gene for SID-1 was amplified from cDNA of *Caenorhabditis elegans* strain N2, kindly provided by Dr. Libusová (Charles University, Faculty of Science, Department of Cell Biology) using 2 primer pairs (for the first part 5´-GTTCACAATGATTCGTGTTTATTTGAT-3´, 5´-AGCATTTGGCCATGGAGTGA-3´ and for the second part 5´-TCACTCCATGGCCAAATGCT-3´ and 5´-TGAAAAACCGGATAGGGA AAACAA-3´). Concurrently 5´-part of *CeSID-1* was modified with the plant signal sequence from *HyPRP* gene (see Dvořková et al., 2012 and sequence in Attachments 9.5.) by classical molecular techniques. The sequence of *SAG18* was amplified by RT-PCR from cDNA of *A. thaliana* ecotype Col-0 using primer pair (5´-TCTCATCTCTCTCTGAAGTAG-3´, 5´-ATCTACCGAGTCTCTTCGAC-3´). The presence of transcripts for both introduced genes in super-transformed GRED213 line was checked by semi-quantitative RT-PCR using primer pairs listed above (data not shown).

We also checked proper protein localization SAG18 and SID-1 by their fusion with *E-GFP*. Binary vectors with c-terminal *E-GFP* fusions and "GASQA" linker were prepared for both proteins (Chapter 3.1. and Attachments 9.5.) and transformed into the BY-2 wt line. Two novel BY-2 lines were derived after selection – SAF (*AtSAG18::E-GFP* fusion) and CEF (*CeSID-1::E-GFP* fusion; see Chapter 3.1.; Fig. 3.1b-**c**, **d**; Tab. 3.1). A confocal microscopy, thereafter, confirmed proper localization of both proteins in comparison with GREEN1 line (Chapter 3.1.; Fig. 3.1a-**a** Tab. 3.1) with free cytosolic GFP (Fig. 4.4.4.2a-**a**, **b**) and *NtRGS::rsGFP* line with a membrane localization (kindly provided as a positive control by Mgr. Šonka; Fig. 4.4.4.2a-**c**, **d**). Both newly introduced proteins (AtSAG18 and CeSID-1) were localized during exponential phase of growth in the plasma membrane (Fig. 4.4.4.2a-**e**, **g**, **i**, **j**), while in the starving stationary cells mostly in the vacuole (but, it should be noted that we observed such a localization also in the case of other plasma membrane-localized proteins; Fig. 4.4.4.2a-**f**, **h**).

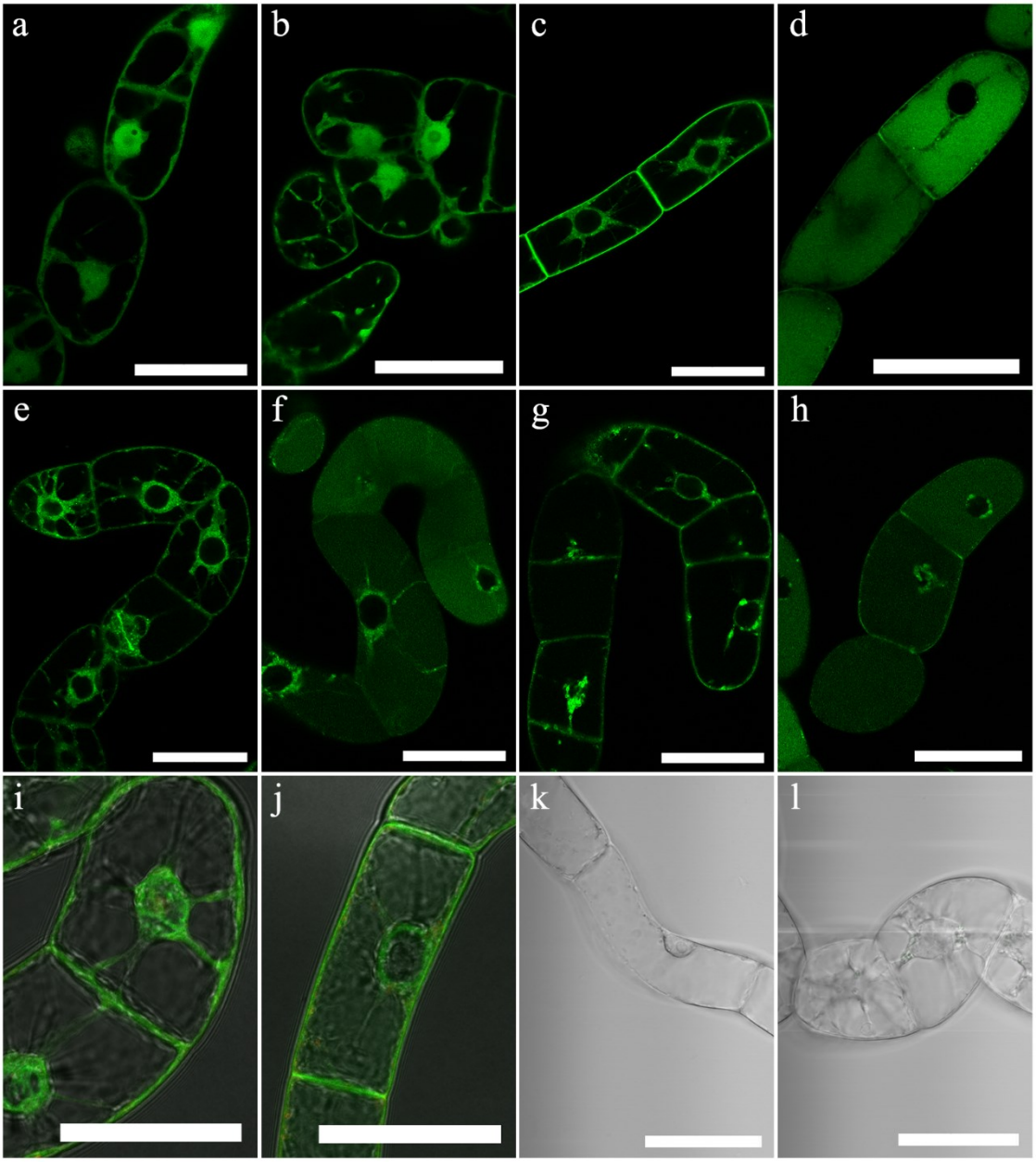


Fig. 4.4.4.2a Localization of AtSAG18 and CeSID-1 E-GFP fusions in tobacco BY-2 cell line in comparison with controls. Confocal pictures were captured from LSM 880 (Zeiss) using filters for GFP (**a**-**l**) and Nomarski contrast (**i**-**l**), processed by ZEN lite (Zeiss) and Adobe Photoshop CS6. Scale bars are 50 µm. (**a**, **c**, **e**, **g**, **i**, **j**, **k**) represent cells in exponential phase, rest in stationary phase; (**a**, **b**) imagine free cytosolic rsGFP (line GREEN1 Chapter 3.1.); (**c**, **d**) *NtRGS::rsGFP* fusion; (**e**, **f**, **i**) show SAF line (*AtSAG18::E-GFP*; Chapter 3.1.); (**g**, **h**, **j**) represent CEF line (*CeSID-1::E-GFP*; Chapter 3.1.) and (**k**, **l**) show wt.

When the proper localization of AtSAG18 and CeSID-1 in our heterologous system was verified, we prepared an experiment testing the possibility of sRNAs movement into protoplasts of 3 different BY-2 lines – GRED213, GRSA33 and GRCE1. By this complex approach we tried to overcome the most problematic points mentioned in the introduction of Chapter 4.4.4.

Protoplasts were prepared from three different lines (GRED213, GRSA33 and GRCE1) and treated with sRNA solution against *GFP* according to Chapters 3.7. and 3.8. Potential changes in the expression of *GFP* and *RFP* were evaluated by qRT-PCR as described in Chapter 3.4 with elongation factor *EF1α* as an internal standard.

GRED213 "mother" line showed the expected reduction of *GFP* transcripts 24 h after the treatment compared with time 0, but completely opposite reaction 6 h after the treatment (Fig. 4.4.4.2b-**a**). *RFP* transcription was mostly reduced in time 0 (Fig. 4.4.4.2b-**b**). When we compared the ratio of both fluorescent protein transcripts, it was growing in favor of the *GFP* mRNA (Fig. 4.4.4.2b-**c**). Transcription of *RFP* and *GFP* in BY-2 line with overexpressed *CeSID-1*, GRCE1, was increasing in time. Exposure to sRNAs caused higher transcription of both *GFP* and *RFP* (Fig. 4.4.4.2b-**a, b**). *GFP* transcription in GRSA line 6 h after the treatment was descendent, but this trend disappeared 24 h after the treatment (Fig. 4.4.4.2b-**a**). *RFP* transcription was more or less the same independently of the treatment (Fig. 4.4.4.2b-**b**).

We further decided to examine the effect of the presence of a proven or putative sRNAs/dsRNA transporter by normalization the data of line GRED213, which should show higher *GFP* transcript levels (lower silencing) compared to the other two lines if SID-1 or SAG18 acted as functional sRNA/dsRNA importers. The level of *GFP* was declining 6 h after the treatment in GRCE and GRSA lines, supporting our hypothesis, but after 24 h trends were totally inverse (Fig. 4.4.4.2c-**a**). Also, the level of *RFP*, used as an internal standard, showed high differences after the treatment which we did not expected considering the treatment with RNAs isolated from the line expressing *GFP*.

To summarize the results – tobacco BY-2 cell line GRED213 was not reacting at the level of *GFP* transcription to externally added sRNAs against the *GFP*. Moreover, lines overexpressing additional *SID-1* or *SAG18* genes were responding non-homogeneously during monitored 24 h, independently of the sRNAs treatment. Visible alterations in both *GFP* and *RFP* levels likely resulted from random changes in the experimental system, but not because of the movement of GFP derived sRNAs into the cells.

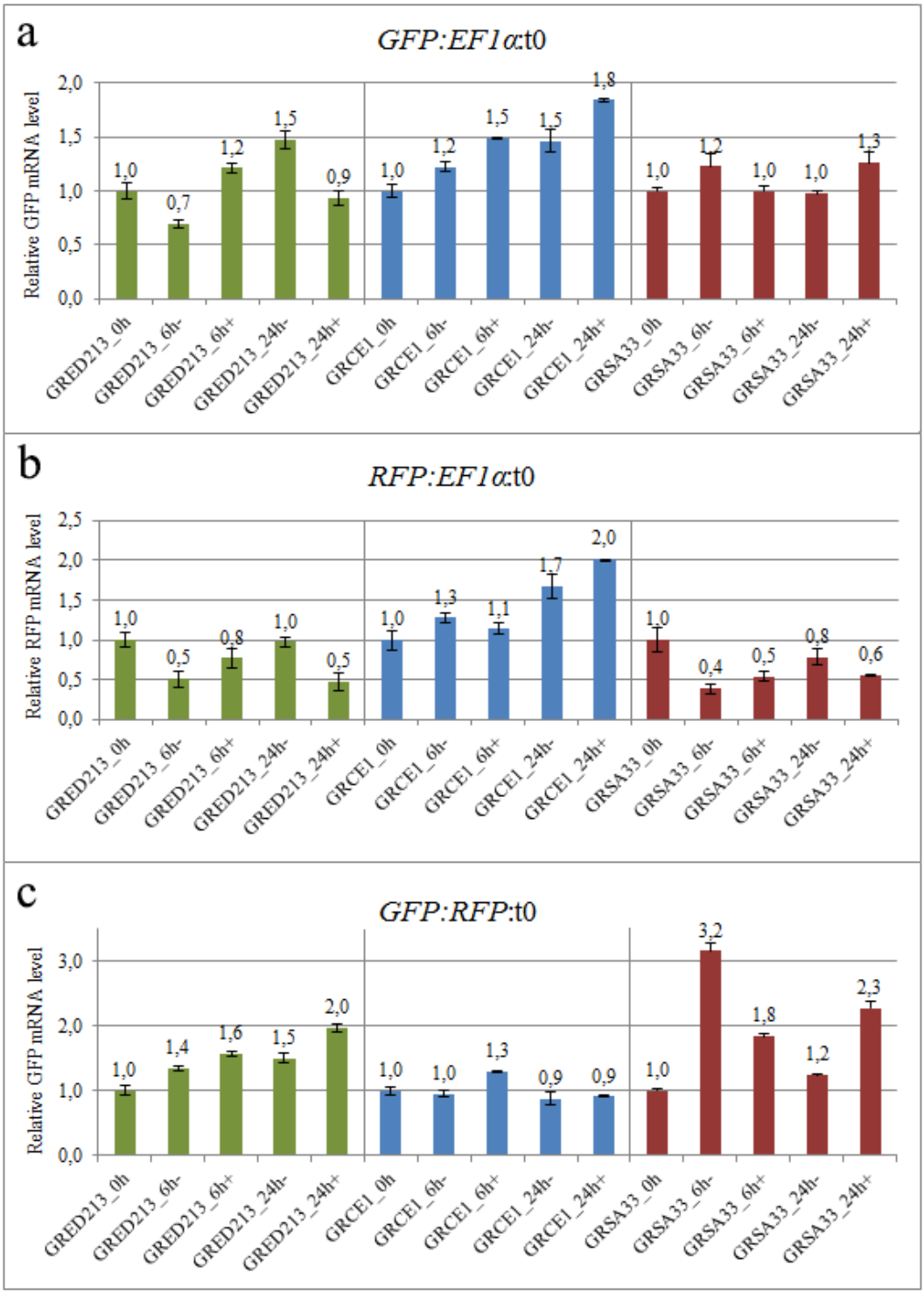


Fig. 4.4.4.2b Time course of *GFP* and *RFP* transcript levels after the treatment with sRNAs against *GFP* (+), measured in protoplasts of three different BY-2 lines by RT-qPCR. The relative transcript levels were related to time 0 that was set to 1.0 and normalized to the internal standard *EF1α* (for **a**, **b**) or *RFP* (for **c**). Error bars in all plots represent standard deviation.

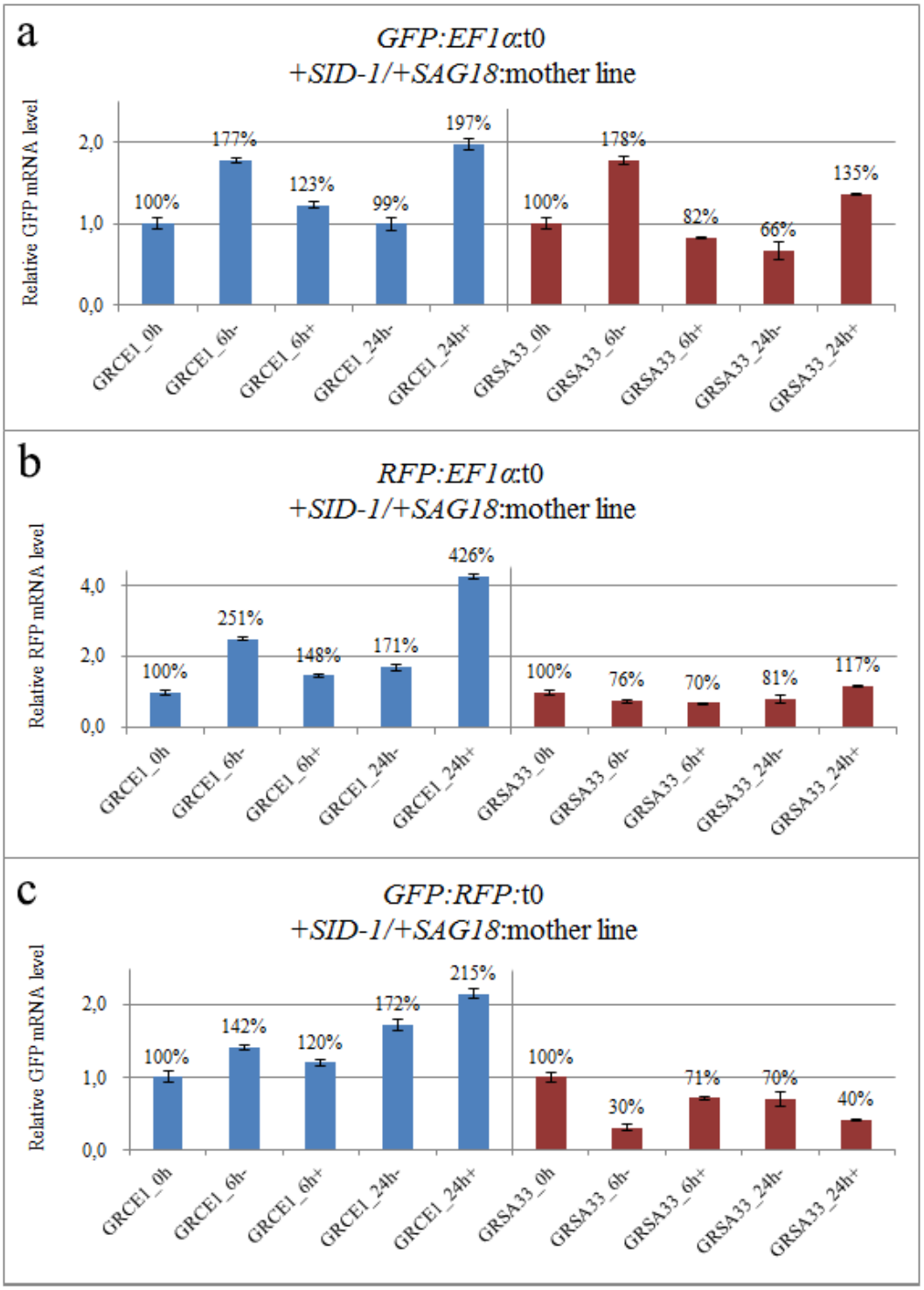


Fig. 4.4.4.2c Time course of *GFP* and *RFP* transcript levels after the treatment with sRNAs against GFP (+), measured in protoplasts of GRSA and GRCE BY-2 lines by RT-qPCR and normalized to GRED213 line. The relative transcript levels were related to time 0 that was set to 100 % and normalized to the internal standard *EF1α* (for **a**, **b**) or *RFP* (for **c**). Error bars in all plots indicate standard deviation.

5. DISCUSSION

The presented work covers a wide range of topics, some of which were studied in collaboration with other colleagues and which are individually discussed in the attached manuscripts. The separate discussion here will therefore focus in particular on several selected topics and raised questions which emerged exclusively during my own research on PhD thesis. Namely, I focus on discussion of the development of suitable methods for studying of transgene silencing, on the usage of appropriate plant model system for visualization of changes provoked by transgene silencing and also on the role of putative transmembrane transporters of sRNAs.

RNA interference is a conserved and very complex mechanism of regulation gene expression in eukaryotic organisms (Baulcombe, 2004). Alpha and omega for this process is the gene silencing through dsRNA formation which leads to the sequence-specific degradation of mRNA, blocking of translation of complementary transcripts or inhibition of transcription by modifications of chromatin. Before the discovery of the mechanism of RNAi (Fire and Mello, 1998), RNA silencing induced by antisense RNA was taken as just an autonomous internal "stoichiometric issue" – it was supposed that the introduced antisense RNA molecule interacted with target mRNA through the Watson-Crick base pairing to prevent corresponding protein biosynthesis (van der Krol et al., 1988). Nonetheless, further numerous analysis using more and more sophisticated techniques and various eukaryotic model organisms revealed that this homology-dependent RNA silencing is a part of the world of small non-coding RNAs that is much larger than anyone expected. Gradually, the involvement in the defense against transposable elements was uncovered (Henderson and Jacobsen, 2007) as well as the role of RNA interference in the regulation of internal or external genes (Eamens et al., 2008). This opened extensive reverse and forward genetic approaches. Nowadays we know that RNAi plays also pivotal role in the whole body development, the response to abiotic or even biotic stresses and in the formation of additional information level in establishing and maintaining chromatin organization (Law and Jacobsen, 2010).

5.1. Monitoring of transgene PTGS switch to TGS needs deeper analysis

The preparation of genetically modified plants became during last 40 years a routine matter for many different species, although some groups of crops were challenging due to difficulties in establishment of stable and regenerative somatic tissue culture (Cheng et al., 1997; Sidorova et al., 2019). Nevertheless, the regular usage of genetically modified plants in basic or applied research was always endangered by unpredictable and unstable level of transgene expression caused by many factors (Baulcombe, 2019) like the random character of T-DNA integration (Kim

et al., 2007), the T-DNA nature itself (De Bolle et al., 2003) or the number of integrated T-DNA copies (Tang et al., 2007), but nearly always tightly interconnected with the RNA interference.

These findings were crucial also for my starting point, when I continued in the work of Nocarova et al. (2010). They observed successive spontaneous silencing of tandem reporter genes in vegetatively propagated potato (*Solanum tuberosum*) plants, even more than four years after the transformation event. Assessment of the T-DNA copy number (by Southern hybridization) showed that lines with small copy number have weaker tendency to spontaneous silencing in agreement with Tang et al. (2007). Their further analysis of *GFP* and *nptII* expression at the transcript (semi-quantitative RT-PCR) and the protein levels (immunodetection on Western blots) over a long period of time revealed that silencing of the two reporter genes differed (Nocarova et al., 2010). Results indicated that the *GFP* was first silenced at the PTGS level in some lines which later developed to the TGS in agreement with the hypothesis already formulated by Fojtova et al. (2003). In contrast, the *nptII* seemed to be silenced directly at the TGS level. This was supported by assessing the effects of AzaC treatment of lines showing spontaneous silencing. AzaC treatment inhibits cytosine methylation (Santi et al. 1984) that is needed to maintain TGS (Fojtova et al., 2003), so this treatment can serve as an indirect method distinguishing PTGS and TGS (read more in Chapters 1.4.1. and 3.2.). However detailed analysis of methylation pattern and precise monitoring of both genes at the transcription level supporting or refusing this hypothesis was missing.

In the follow-up study (Tyč et al., 2017) we optimized the AzaC treatment of potato leaf explants from lines with transcriptionally silenced transgenes. We succeeded to reactivate silenced transgenes at the whole plant level in comparison with studies, where they restored transcriptionally silenced genes only at cell, organ or tissue levels (Wang and Waterhouse 2000; Emani et al., 2002; Kanazawa et al., 2007; Tyunin et al., 2012). Nevertheless, it should be noted that in case of GFP we observed very often only temporary restoration of expression, seen only in calli, that indicated re-silencing of the transgene during subsequent *de novo* regeneration. Both transgenes were restored only in 30 % of regenerants derived from the line R17 bearing multi copy T-DNA insertions, while regenerants of R28 line (supposed to carry a single copy insertion) restored only *nptII* gene at the whole plant level. Obtaining lines with reactivated *nptII* was made possible by careful comparison of the promoter methylation via bisulfite modification, confirming that the silencing was at the transcriptional level. At the same time, it turned out that the R28 line was not present as a single copy, but probably double copy, and only one insertion was reactivated by the demethylation.

These results support the hypothesis that both *Pnos* and *nptII* methylation dramatically declined after AzaC treatment, but only in some T-DNA insertions. Some studies depicted

the stability of T-DNA methylation pattern in time (Kunz et al., 2003), contrary others pointed out the dissemination of DNA methylation after transposon insertion (Martin et al., 2009). However, neither detailed analysis of cytosine methylation in *Pnos* nor *nptII* could answer the question, if methylation had been spreading upstream into the *nptII* cassette from the methylated *GFP* cassette.

5.2. Inducible system together with precise sRNAs analysis allow description of PTGS in detail

In the experiments with potato plants mentioned above, an important phenomenon of spontaneous silencing at the whole plant level was studied. However, this system was inappropriate for an accurate study of the onset of PTGS and the spread of related methylations in time. Therefore, we switched to another important plant model – tobacco BY-2 cell line, with many advantages mentioned above that enable more detailed analysis (Nagata et al., 1992; Srba et al., 2016). We decided to use it in a combination with the system for inducible expression of silencing RNAs. The utilization other advanced high value-added techniques, such as flow-cytometry, qRT-PCR and parallel sequencing of sRNAs, allowed to describe the initial phases of PTGS, but also to better understand the events observed in potatoes, such as PTGS switch to TGS or the connection between *GFP* silencing and follow-up *nptII* silencing.

In our study (Čermák et al. submitted), we provoked PTGS of *GFP* by the production of sRNAs that dated back to different source RNAs. Flow-cytometry and qRT-PCR data showed that the speed and the strength of silencing were highly dependent on the origin and amount of sRNAs (supported by sRNAs sequencing). The most effective PTGS was induced via an inverted repeat construct (IR) producing hairpin RNA, in agreement with Wang and Waterhouse (2000) or Wesley et al. (2001). This type of silencing is frequently present also in endogenous genes and their homologues *in trans* (Muskens et al., 2000) with dominance of 21 nt sRNAs (Yoshikawa et al., 2005; Zicola et al., 2019), consistent with data receiving from expression of the *P35S* hairpin (Přibylová et al., 2019). However, in IR-induced PTGS of *GFP*, a dominant group of sRNAs was 22 nt in length, which was also noticed by Dalakouras et al. (2019). DNA methylation was also observed in unterminated (UT) variant, but it was not found in variant with antisense orientation (AS). Concurrently, we did not observe the spreading of methylation upstream to the *P35S* promoter in any variant, as we supposed in studies with potato plants. When we looked closer to the distribution of sRNAs (which are necessary for DNA methylation) along the target or silencer T-DNAs for all variants we did not find any significant number of sRNAs against the *P35S*. Interestingly in AS and UT variants there were frequent transitive sRNAs against *Tnos* terminator, which lies downstream of the *GFP*, as well as *hpt* and

nptII resistance genes. Investigation of 5' transitivity in these resistance genes showed sRNAs only from the *hpt*, but not from the *nptII* gene with the same terminator. The two silenced tandem transgenes (*GFP*, *nptII*) studied in potato also shared the same *Tnos* terminator. So, it is possible that the methylation of *nptII* gene and its promoter (*Pnos*) in our study Tyč et al. (2017) could be caused by transitivity spreading from the *Tnos* terminator via sRNAs. However, both mentioned studies (Čermák et al. submitted) and Přibylová et al. (2019) showed very high preciseness of methylation with very small spill-over outside of the targeted sequence in the short-term exposure to sRNAs – 14 or 10 days, respectively. Such a DNA methylation outside of the target sequence was also observed in short flanking regions in the T6 generation of the *A. thalina* transgenic line without apparent long-range spread (Zicola et al., 2019). It should be also noted that both methylation analysis did not cover the whole target and inductor T-DNAs, including *nptII* and *hpt* gene (Přibylová et al., 2019; Čermák et al. submitted). Therefore, we don't know if the sRNAs aligning to the *hpt* gene were enough to cause the methylation.

Another question remains – why we did not observe also sRNAs against *nptII* gene which share the same terminator as *hpt* gene? We can assume that these differences were caused by the distinct nature of sequences situated upstream of the *Tnos*. However the presumptive spreading of methylation from transcribed region into the promoter in research with potatoes (Nocarova et al., 2010; Tyč et al., 2017) had much longer time (months to years) comparing the situation observed in tobacco cells (Přibylová et al., 2019; Čermák et al. submitted) or in Arabidopsis (Zicola et al., 2019). It should also be borne in mind that we used two different plant systems and cell differentiation and the whole plant context could also play an important role in the methylation processes observed in potatoes, as confirmed by study Weinhold et al. (2013). To elucidate the possibility of upstream methylation spreading in the tandem arrangement of transgenes, it would be suitable to monitor the methylation of both the target and the inducer T-DNAs over a longer time horizon, including sRNAs transitivity analysis. The possible effect of sequence differences between terminators and resistance genes on the spread of methylation could be revealed by the modification of T-DNAs by unifying sequences of the downstream resistance gene. Arrangements with different terminators could also bring interesting results.

5.3. Use of potato lines prone to silencing

Our analyses on potato identified transgenic lines that showed silencing of the reporter genes at the TGS level and whose expression could therefore be restored by AzaC treatment (Nocarova et al., 2010; Tyč et al., 2017). We hypothesized that plants regenerated from cells with AzaC reactivated transgene expression had larger tendency to be spontaneously silenced again, because the T-DNA insertions present in these lines had been silenced spontaneously once. If we found

conditions that would trigger the silencing in such susceptible lines, such treatment could be used for early preselection of newly prepared transgenic lines to remove those that are prone to spontaneous silencing during subsequent cultivation. We found that unlike other treatments, regeneration *de novo* from leaf segments could be potentially used for this purpose because about 10 % of shoots regenerated from leaves of reactivated plants exhibited re-silencing. Our genetic material with proven tendency in spontaneous silencing thus represented a valuable tool that helped us to find that *de novo* regeneration *in vitro* stimulates transgene silencing. It is not a big surprise considering dynamic epigenetic changes during dedifferentiation and redifferentiation processes (Brettell and Dennis, 1991; Weinhold et al., 2013; Lee and Seo, 2018). However, it is absolutely necessary to test this method also on other plant models, since Weinhold et al. (2013) observed in contrast to us the restoration of transgene expression during *de novo* regeneration in *Nicotiana attenuata.*

5.4. The key role of sRNAs in RNAi and their systemic movement

Previous studies on the tobacco BY-2 cell line, whether focusing on the effect of sRNAs of various origins on PTGS dynamics (Čermák et al. submitted) or the initial phase of TGS (Přibylová et al., 2019), have shown the great importance of detailed analysis of sRNAs, as key players in RNAi (Hamilton and Baulcombe, 1999). We were particularly interested in their length, amount and distribution with respect to the target sites and their relation to DNA methylation. It turned out that next generation sequencing methods can provide such data with high information value (Fehlmann et al., 2016). However, the investigation of the involvement of these small RNAs in the processes distant from the site of their formation far exceeded the scope of the above works. Progress in the discovery of small RNA mobility is briefly described in the introductory chapter of this thesis. Here, I would rather focus on the interesting aspects and unexplored areas that led to our searching for specific transmembrane transporters of small RNAs in plants.

Early studies, since the very first description of RNAi in *C. elegans* (Fire et al., 1998), have suggested that a silencing signal in the form of dsRNA may spread between nematode tissues or even pass through the gut wall from digested bacteria (Timmons and Fire, 1998). But the mechanism was unknown until 2002, when Winston and colleagues discovered the transmembrane protein responsible for the systemic RNAi, SID-1. In plants, the systemic nature of RNAi has been studied mainly in connection with plant transformation (Voinnet et al., 1998), virus-induced gene silencing (VIGS; Jones et al., 1999), grafting experiments (Palauqui et al., 1997) or even plant-pathogen interactions (Weiberg et al., 2013). Over time, it has been shown that the silencing signal in the form of sRNAs is transported between cells through plasmodesmata for the short-distance movement and via phloem for the long distance systemic movement

(Voinnet et al., 1998). However, very few studies have questioned if and how sRNAs are transferred between cells that are inherently free of plasmodesmata (e.g. stomata guard cells or vegetative nucleus and sperm cells in pollen; Voinnet et al., 1998; Himber et al., 2003; Slotkin et al., 2009) or how sRNAs are transferred from/to the external environment (Himber et al., 2003; Voinnet, 2005; Patton et al., 2015; Pyott and Molnar, 2015). Some hypothesized transmembrane-associated transporters and receptors (Pyott and Molnar, 2015), others favored secretion through vesicles (Cai et al., 2018). Together with the observation described in the introduction to Chapter 4.4. on spontaneous silencing of GFP after mixing two tobacco lines, we decided to search for and to investigate the presence of a plant homolog of the animal dsRNA transporter protein SID-1.

5.5. *SAG18* and *aPHC* as putative homologs of SID-1

Comparing of protein sequences for *C. elegans* SID-1 and *D. discoideum* SID-1 from another phylogenetic branch of Amoebozoa with a plant sequences database, we found a very weak putative plant homologue of these proteins in *A. thaliana*, Senescence Associated Gene 18 (*SAG18*; Fig. 4.4.1c; Miller et al., 1999). The function of *SAG18* is unknown, but *SAG18* orthologues are conserved across the embryophytes (see Chapters 9.2. and 9.3.). Due to the highly probable gene duplications in plants during the evolution (summarized in Soltis et al., 2014), a candidate paralog in *A. thaliana*, putative alkaline phytoceramidase (*aPHC*; Tabata et al., 2000), was found in the database. Although both proteins showed very low sequence similarity to SID-1, predicted transmembrane localization of SAG18 and aPHC supported the hypothesis of relatedness (Fig. 4.4.1a and Chapter 9.1.). The transmembrane localization of *AtSAG18* was also confirmed *in vivo* in transformed tobacco line BY-2 by its fusion with *GFP* (Fig. 4.4.2a). However, it should be noted that SAG18 and aPHC lack the 5´-rich region of SID-1, which also contains some key residues for protein functionality in animal systems (see Fig. 4.4.1a and 9.1.; Li et al., 2015). On the other hand, some key residues, such as Ser-536, are consistent with SID-1 in both plant proteins (Chapter 9.1.; Li et al., 2015). Over the years, other nematode proteins have been discovered that are involved in the transport of small RNAs between different tissues or from the external environment – SID-2 (McEwan et al., 2012), SID-3 (Jose et al., 2012) and SID-5 (Hinas et al., 2012), which together form the vesicular transport system with receptor kinases and transmembrane transporters (Fig. 1.6b; Rocheleau, 2012). Comparison of their protein sequences with plant databases revealed only partial structural homology, e.g. for SID-2 in plant PIRLs (plant-specific family of leucine-rich repeat proteins; data not shown).

Other indicia suggesting SAG18 involvement in sRNA transport were expression profiles of *SAG18* and *aPHC* in *A. thaliana* in the Genevestigator database (Fig. 4.4.1d). Both proteins showed a high level of expression in many tissues and especially in sperm cells or stomata guard

cells, where we expected their increased participation in the transport of sRNAs across membranes due to the absence of plasmodesmata (according to Voinnet, 2008; Himber et al., 2003; Slotkin et al., 2009). Mutant plants with a double mutation in both candidate proteins were prepared, but no visible change of phenotype was observed even during several generations of self-fertilization (Fig. 4.4.2a, b). We assumed, when the SAG18 and aPHC proteins might be involved, for example, in ensuring inactivation of TEs in generative cells, as suggested by the study of Slotkin et al., 2009 (Fig. 1.5.1). Thus, possible changes could manifest themselves with an increasing number of generations. Similarly, in the transfer of methylation pattern via small RNAs to generations (Zicola et al., 2019).

Since the participation of small RNA transporter proteins in host-induced gene silencing (HIGS) interactions can also be expected, we also tested effects of mutations in the candidate genes on the degree of susceptibility to *Botrytis cinerea* (Weiberg et al., 2013). However, there was no significant difference in the degree of pathogen damage in *sag18 aphc* double mutants or wt plants (Fig. 4.4.2d). Until recently, the group of Cai et al. (2018) found two *A. thaliana* genes, tetraspanin-like 8 and 9, that are important in silencing of *B. cinerea* virulence genes. Simultaneously, they showed that sRNAs are transported to the fungal host via extracellular vesicles and not directly through membrane transporters as we supposed. However, this vesicle-mediated transport of sRNAs between plant and fungal cells still does not contradict the existence of special transmembrane transporters of RNAs for in planta communication, given the situation described above in *C. elegans*. Together with findings about possible role of ceramides in endosomal transport by Trajkovic et al. (2008), it might be possible the coupling of special transporters together with vesicular transport (as aPHC is annotated as putative ceramidase). However, it is also possible that the SAG18 and aPHC proteins may be involved in other transport or signaling processes, as suggested by a pilot gravimetric comparison of the activity of stomata guard cells in the double mutant and wt plants (Fig. 4.4.3). Further study could focus on more detailed analyzes of this hypothesis, including other known *A. thaliana* mutants affected in different processes involved in stomata closure and regulation of their activity (Eisenach and De Angeli, 2017). Due to the change of my research focus towards applied research in different institute, these analyses could not be performed because of time constraints.

5.6. Testing of possible transmembrane transport of sRNAs in tobacco BY-2 line

The use of tobacco BY-2 cell line, albeit to some extent an artificial system, to monitor the non-symplastic (transmembrane) transport of small RNAs in plants was based on the observation of spontaneous silencing of GFP in various mixed transgenic lines (Nocarova et Fischer, unpublished). To facilitate the investigation, a system of two transgenic lines was created, where

one served as a donor of sRNAs against *GFP* and the other as their acceptor carrying *GFP* and at the same time *RFP* in the tandem arrangement. The donor became the IR8C line carrying two different T-DNAs – i) *P35S::GFP* and ii) the cassette for *GFP* as inverted repeat under estradiol inducible promoter (Čermák et al. submitted). Induction of this line with estradiol showed a significant reduction in GFP expression within three days, suggesting massive production of sRNAs (Fig. 4.4.4b). The legitimacy of our choice was then confirmed by the detailed sequencing analysis confirming presence of sRNAs against *GFP*, especially from the central and 5' regions of the gene and the predominant length 22 > 21 > 24 nt (Čermák et al. submitted). A number of studies suggest precisely this length of sRNAs as being suitable for transport minimally by the phloem (Yoo et al., 2004; Buhtz et al., 2008; Molnar et al., 2010; Kehr and Kragler, 2018).

In the first phases of the study, we tried to mix two lines described above without obvious consequences for *GFP* expression in the acceptor line (data not shown). However, if we assume that *SAG18*, whose expression we confirmed in BY-2 (Fig. 4.4.4a), is a homologue of *SID-1*, an dsRNA importer in animals (Shih and Hunter, 2011), then sRNA may not be released effectively from donor cells. Therefore, for further experiments, we harvested and frozen the donor suspension after induction in liquid nitrogen and assumed that the cells were disrupted during thawing and their contents spilled out, including sRNAs. However, to avoid possible RNA lability due to RNase activity (Garcia et al., 2017), we used a total extract of all RNAs, simultaneously enriched in the low molecular weight (LMW) RNA fraction (Rosas-Cárdenas et al., 2011) and finally stabilized with the RNase inhibitor protein, RiboLock. Although some studies indicate considerable stability of mobile forms of sRNA in animal extracellular fluids (Knipp, 2014), we preferred a conservative approach and sought to protect sRNAs, recognizing that other studies suggest protection of extracellular RNA by membrane vesicles (Patton et al., 2015; Cai et al., 2018). It also offered the opportunity to test the application of other transportable nucleic acid forms, such as 18-22 nt long antisense oligodeoxynucleotides (AODN). These are now successfully used to study the role of genes especially in the germinating pollen tubes (Bezvoda et al., 2014), but modifications of its backbone appear to adversely affect the transportability by specific carriers such as SID-1 (Shih et Hunter, 2011).

To seek changes in GFP expression, we finally chose qRT-PCR (Gachon et al., 2004) for precise monitoring of transcript level. When setting up the experiment to treat BY-2 transgenic lines with the isolated RNA extract, we decided also to use lines overexpressing *A. thaliana SAG18* gene and *C. elegans SID-1* as additional controls. The proper membrane localization of both proteins was confirmed in tobacco BY-2 lines by the fusion with *E-GFP* (Fig. 4.4.2a). By including the *SID-1* variant, we tested also the functionality of the animal RNA transporter in plant cells, which to our knowledge, has not yet been tested in the plant system.

The functionality of *C. elegans SID-1* in heterologous animal systems, like the expression of *C. elegans SID-1* in mouse embryonic stem cells (Tsang et al., 2007), *Bombyx mori* cells (Xu et al., 2013) or even *Drosophila* cells (Feinberg and Hunter, 2003), facilitated the passive uptake of dsRNA into the recipient animal cells (Feinberg and Hunter, 2003). At the same time, it is surprising that *Drosophila* lacks a functional homolog of *SID-1*, but the presence of nematode *SID-1* facilitates dsRNA uptake (Winston 2002). Nevertheless, in our system with tobacco protoplasts, we were unable to prove the effect of the addition of RNAs on the transcript level of target gene (Fig. 4.4.2b). It would be appropriate to check the stability of the RNA transcript over time by qRT-PCR and its functionality also in animal systems, such as nematodes. The character of the RNA extract could also play a role, because *SID-1* is known for its affinity towards dsRNA as shown in Winston et al. (2002), who successfully used 100 bp dsRNA in their study on *Drosophila*. However, high levels of dsRNA cannot be expected in our total RNA extract due to the activity of DCL proteins.

The design of our experiment did not allow to directly monitor the internalization of RNAs into BY-2 cells, only their potential impact on the reporter gene transcription. Therefore, it is also possible that RNAs were internalized, but their amount in the cells was not high enough to induce changes that could be seen at the level of transcription. Such a dependence on exceeding the threshold level was indicated by the binary nature of silencing during very low induction that we observed in the study of (Čermák et al. submitted). The RNA sequencing data from this study (obtained after the end of experiments with sRNA transmembrane transport) also showed that PTGS can be accompanied with the formation of transitive sRNAs from the *Tnos* terminator. Since this region was used for both *GFP* and *RFP* cassettes in our system, such terminator-specific sRNAs could also negatively affect the expression of primarily non-targeted *RFP*. However, it should be noted that these sRNAs occurred only when PTGS was induced by antisense RNAs and RNAs without terminator, but not by the inverted repeat variant which was the case of the IR8C acceptor line. Even so, it would be necessary to modify the target T-DNA and use at least different terminators for possible future studies. So, considering all aspects, it seems more likely that the role of SAG18 could be different, as suggested by the study of stomata guard cells (Chapter 4.4.3.). It is also possible that plants do not have any specialized transporter of sRNAs, e.g. due to the nature of the cell wall.

6. SUMMARY

During this doctoral thesis, the findings of two scientific publications, one submitted manuscript and also the original yet unpublished results concerning selected mechanisms of RNA interference in plants were summarized. Specifically, the possibility of restoration the expression of silenced transgene at the transcriptional level and testing the susceptibility of potato lines to spontaneous silencing (Tyč et al., 2017), the initial phases of silencing at the transcriptional (TGS; Přibylová et al., 2019) and posttranscriptional (PTGS; Čermák et al. submitted) level. An important part is also the outputs devoted to the possible transmembrane (non-symplastic) transport of small RNAs (sRNAs). Here I will focus mainly on those results that were closely related to my dissertation.

6.1. The expression of previously silenced transgenes can be restored at the whole plant level

The results of the first publication showed that short-term exposure of transgenic *Solanum tuberosum* explants to the demethylating drug 5-azacytidine can restore the expression of transcriptionally silenced transgenes. This can lead to generation of whole plants with reactivated expression when 5-azacytidine treatment is combined with *de novo* regeneration. In the studied potato lines, transcript analyzes, and promoter methylation confirmed a temporary or permanent restoration of the studied transgenes expression. It has been shown that plants with reactivated expression of the transgenes of interest still have an increased tendency to spontaneous silencing again. This occurred to an increased extent, especially during *de novo* regeneration from leaf segments. It is therefore possible to use this method as a "trigger" for silencing in newly derived transgenic lines in order to eliminate susceptible ones. Unfortunately, the potato model organism did not allow optimal observation of the transition from PTGS to TGS and the possible association between the spread of methylation within the tandem arrangement of both monitored transgenes.

6.2. The nature of posttranscriptional silencing depends on the origin of small RNAs

In the second publication, we deliberately induced PTGS with three different estradiol-inducible constructs in the *Nicotiana tabaccum* BY-2 cell line. Detailed analysis of the expression of the silencing-targeted transgene showed a dramatic decrease during the first days after induction, especially in the case of silencing induced by inverted repeat (IR). Parallel sequencing revealed high levels of specific sRNAs. IR-induced silencing was also accompanied by extensive methylation of the target region, which, however, did not spread to the promoter and, apart from CG methylation, decreased after inducer removal. The transition from PTGS to TGS did not occur

in such a short time horizon. An interesting finding was the high level of transitive sRNAs against the *Tnos* terminator observed in lines with antisense or unterminated *GFP*-induced PTGS. This suggests an alternative way of spreading methylation along the T-DNA, different from what we expected from our potato studies.

6.3. The methylation of promoter induced by small RNAs is very accurate and rapid

The knowledge of TGS dynamics is also useful to understanding the relationship between PTGS and TGS. In the third publication, we thus specifically induced TGS in the BY-2 cell line. The study showed that the massive formation of sRNAs from the promoter sequence leads to the rapid onset of precise methylation of the target promoter and the gradual associated attenuation of downstream transgene expression. However, some sRNAs also came from regions outside the promoter, such as the *Tnos* terminator or the *hpt* gene.

6.4. The plant protein SAG18 and its homologue aPHC probably do not serve to transport sRNAs

For a possible role in sRNA transmembrane transfer, candidate plant proteins SAG18 and aPHC were selected based on homology to the animal dsRNA transporter, SID-1 protein. The study confirmed plasma membrane localization of SAG18 in BY-2 cells. The system of two transgenic lines was also developed to study the possible transmembrane transport of sRNAs by both the plant protein SAG18 and the animal SID-1. The donor line efficiently produced the appropriate sRNAs, but their addition to the protoplasts of the acceptor lines did not alter the expression of the target transgene. *Arabidopsis thaliana* plants double mutated in the *SAG18* and *aPHC* genes did not show obvious changes in phenotype compared to wild type plants, nor did they reveal the effect of mutations on several tested parameters related to the sites of *SAG18* and *aPHC* expression. Only an insignificant trend was observed in the change of transpiration of plants with the double mutation indicating a possible role of the monitored proteins in the activity of the stomata or its regulation.

7. ZÁVĚRY PRÁCE

Během této doktorské práce byly shrnuty poznatky dvou vědeckých publikací, jednoho podaného manuskriptu a též originální dosud nepublikované výsledky věnující se vybraným mechanizmům RNA interference u rostlin. Konkrétně se jednalo o možnosti obnovení exprese transgenu umlčeného na transkripční úrovni a testování náchylnosti linií bramboru ke spontánnímu umlčení (Tyč et al., 2017), počátečním fázím umlčování na transkripční (TGS; Přibylová et al., 2019) a posttranskripční úrovni (PTGS; Čermák et al. podáno). Významný díl představují též výstupy věnované možnému transmembránovému (nesymplastickému) transportu malých RNA (sRNAs). Zde se zaměřím zejména na ty výsledky, které úzce souvisely s vlastní disertační prací.

7.1. Expresi dříve umlčených transgenů lze obnovit na úrovni celých rostlin

Výsledky první publikace prokázaly, že krátkodobé vystavení explantátů z transgenních rostlin bramboru (*Solanum tuberosum*) demetylační droze 5-azacytidinu může obnovit expresi transkripčně umlčených transgenů. V kombinaci s *de novo* regenerací lze takto získat celé rostliny s reaktivovanou expresí. U studovaných linií bramboru analýzy transkriptů i metylace promotoru potvrdily dočasné či trvalé obnovení exprese transgenů. Ukázalo se, že rostliny s reaktivovanou expresí sledovaných transgenů mají i nadále zvýšenou tendenci k opětovnému spontánnímu umlčování. K tomu docházelo ve zvýšené míře zejména během regenerace *de novo* z listových segmentů. Nabízí se tedy možné užití této metody jako „spouštěče“ umlčování u nově odvozených transgenních linií s cílem vyřadit ty náchylné. Bohužel modelový organismus bramboru příliš neumožňoval dostatečně osvětlit pozorovaný přechod PTGS na TGS a možnou spojitost mezi šířením metylace v rámci tandemového uspořádání obou sledovaných transgenů.

7.2. Charakter posttranskripčního umlčování závisí na původu malých RNA

Ve druhé publikaci jsme záměrně navozovali PTGS třemi různými estradiol-inducibilními konstrukty u buněčné linie tabáku (*Nicotiana tabaccum)* BY-2. Detailní analýza exprese transgenu cíleného k umlčování ukázala dramatický pokles již během prvních dnů od indukce, zejména v případě umlčování indukovaného invertovanou repeticí (IR). Paralelní sekvenování odhalilo vysoké hladiny specifických sRNAs. Umlčování indukované IR bylo též provázeno rozsáhlou metylací cílové oblasti, která se však nešířila do promotoru a s výjimkou CG metylace poklesla po odstranění induktoru. K přechodu PTGS na TGS v takto krátkém časovém horizontu nedošlo. Zajímavým zjištěním byla vysoká hladina transitivních sRNAs proti *Tnos* terminátoru u linií, kde bylo PTGS indukováno *GFP* v antisense orientaci či bez terminátoru. Jejich

přítomnost tak naznačuje alternativní způsob šíření metylace podél T-DNA, než jsme předpokládali v našich studiích s bramborami.

7.3. Metylace promotoru indukovaná malými RNA je velmi přesná a rychlá

Pro pochopení vztahů mezi PTGS a TGS je i klíčová znalost dynamiky TGS. Ve třetí publikaci jsme tak cíleně indukovali TGS u buněčné linie BY-2. Studie prokázala, že mohutná tvorba sRNAs z promotorové sekvence vede k rychlému nástupu přesné metylace cílového promotoru a postupnému souvisejícímu útlumu exprese „downstream" transgenu. Některé sRNA však pocházely i z oblastí mimo promotor, například *Tnos* terminátoru či *hpt* genu.

7.4. Rostlinný protein SAG18 a jeho homolog aPHC nejspíš neslouží k transportu sRNA

Pro možnou roli v transmembránovém přenosu sRNA byly na základě homologie s živočišným transportérem dsRNA, SID-1 proteinem, vytipovány kandidátní rostlinné proteiny SAG18 a aPHC. Studie na buněčné úrovni v BY-2 liniích potvrdila membránovou lokalizaci SAG18. Byl také vytvořen systém dvou transgenních linií pro studium možného transmembránového transportu sRNAs prostřednictvím jak rostlinného proteinu SAG18, tak i živočišného SID-1. Donorová linie efektivně tvořila příslušné sRNAs, jejichž přídavek k protoplastům akceptorových linií však nevyvolal změnu exprese cílového transgenu. Rostliny huseníčku (*Arabidopsis thaliana*) dvojitě mutované v genech *SAG18* a *aPHC* nevykazovaly zjevné změny ve fenotypu ve srovnání s rostlinami nemutantními a ani u nich se nepodařilo odhalit vliv mutací na řadu testovaných parametrů souvisejících s místem jejich výrazné exprese. Pouze byl pozorován nesignifikantní trend ve změně transpirace rostlin s dvojitou mutací, naznačující možnou roli sledovaných proteinů v činnosti průduchů či její regulaci.

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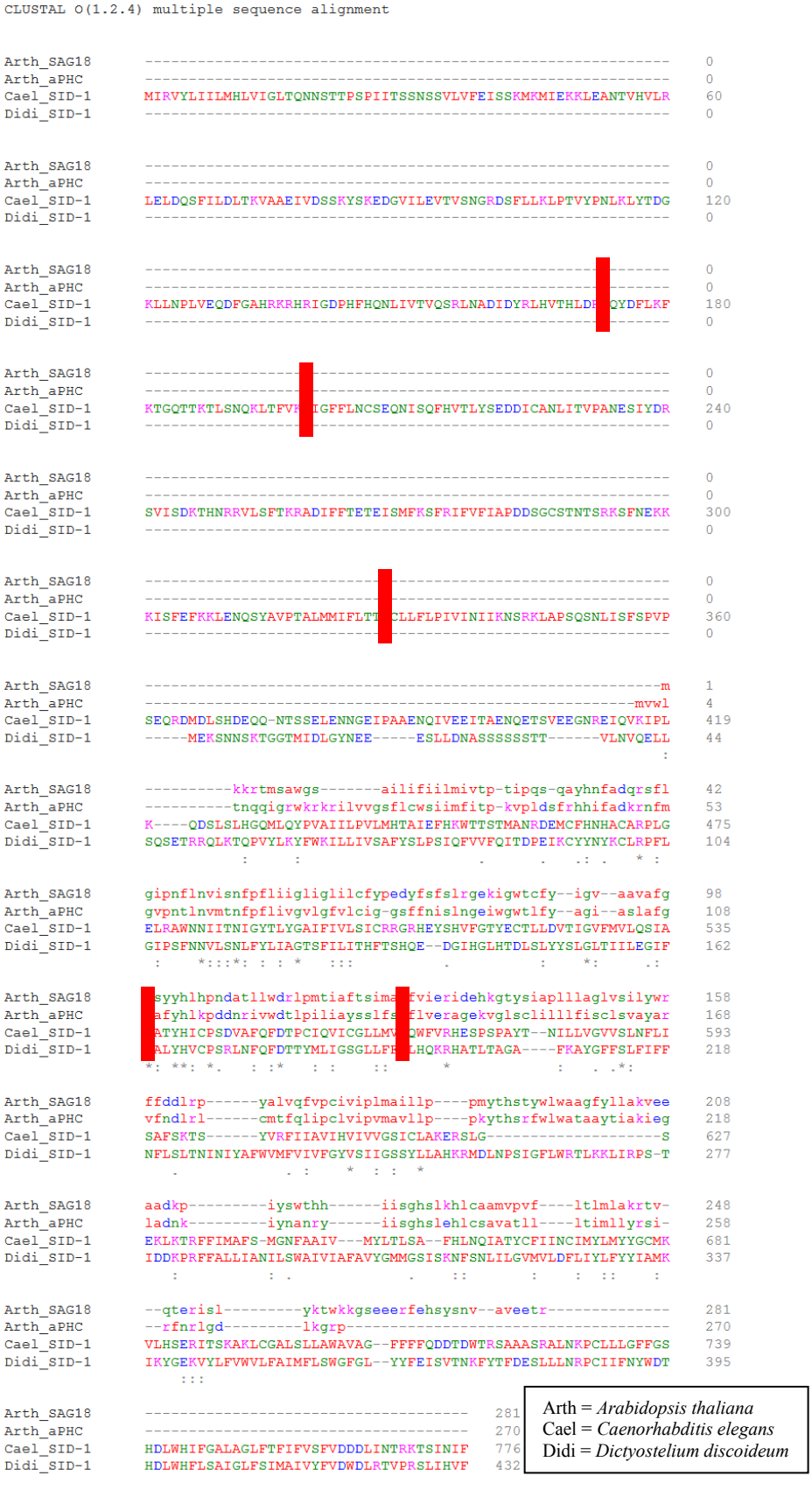
9. ADDITIONAL DATA

9.1. SID-1, SAG18 and aPHC alignment with highlighted key residues of SID-1 from *Caenorhabditis elegans* (red rectangles).



9.2. Alignment of chosen embryophyte SAG18 with highlighted conservative Serin.

9.3. A phylogenetic tree from SAG18 protein sequences from selected genera of Archaeplastida. The tree was constructed using Neighbor-Joining method in Geneious sftw.

9.4. Primers used for genotyping

Tab. 9.4. Primer pairs for *Arabidopsis thaliana* SALK mutant lines genotyping.

primer name	sequence	target	melting temperature
Fw1	5′CCTGAGAATCTCATCTCTCTC3′	*SAG18*	
Rev1	5′AGTAGCATCGTTTGGGTGAAG3′	*SAG18*	
Rev-insert	5′ACAACACTCAACCCTATCTCG3′	*pROK2*	
Fw2	5′CGTAGACGTTTAGAGCGGTC3′	*aPHC*	
Rev2	5′TACACATGCACAGAGACAGAG3′	*aPHC*	

9.5. Gene sequences used in non-published studies

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