Studies on the regulation of Polycomb-target genes and abiotic stress responses by the *Arabidopsis* protein BLISTER

Inaugural-Dissertation

Zur Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf, März 2016

aus dem Institut für Genetik
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Daniel Schubert

Korreferent: Prof. Dr. Rüdiger Simon

Tag der mündlichen Prüfung: 02.05.2016

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Ort, Datum Unterschrift

Hofstadter's Law:

It always takes longer than you expect,

even when you take into account Hofstadter's Law.

Publications resulting from this work:

Kleinmanns, J.A., and Schubert, D. (2014).
Polycomb and Trithorax group protein-mediated control of stress responses in plants. Biol Chem *395*, 1291-1300.

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1 Introduction

1.1 Epigenetics

In eukaryotic organisms the macromolecule deoxyribonucleic acid (DNA) encodes the genetic information that is essential for the development of any organism. Heritable changes in the DNA sequence, which are propagated to the next generation via the germ line, are the driving force of evolution. During the development of organisms, different genes become activated or repressed according to the developmental stage and the respective tissue. Activation and repression of genes is correlated with binding of transcriptional activators and repressors, so-called transcription factors (TFs), to promoter regions of target genes. Not only binding of TFs is fundamental for the question whether a gene is transcribed. DNA methylation or modifications of DNA associated histone proteins also result in altered gene expression. These so called 'epigenetic' modifications, e.g. addition of different residues to the DNA or to histones leading to either activation or repression of a gene, are by definition 'mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence' (Russo et al., 1996), and can be reversed in accordance to developmental or environmental requirements. Regulatory processes such as imprinting, silencing of transposable element expression and mobility, parent-of-origin effects, X-chromosome inactivation, reprogramming, positional effects and others are considered 'epigenetic' processes. The term 'epigenetic' does not only include modifications of the DNA and histones, it also refers to chromatin-remodeling, exchange of histones or nucleosomes and also RNA based mechanisms, leading to altered, heritable gene expression patterns. An unusual form of epigenetics are prions, proteins that self-propagate changes in their folding which are by definition stable during mating and meiosis and thus allow transmission trough the germ line (reviewed in Halfmann and Lindquist, 2010).

1.1.1 Chromatin

In eukaryotes the DNA does not lie 'naked' in the nucleus but is associated with many proteins resulting in cytologically visible chromatin. To fit the huge DNA molecule into the nucleus it needs to be organized and compacted. This is achieved by wrapping of ~147 bp DNA around an octamer of histone proteins. These octamers are composed of each two copies of the core histones H2A, H2B, H3 and H4 and together with the DNA build the so called nucleosome (Luger et al., 1997). Two nucleosomes are connected by 20-50 bp linker DNA, making the

oligo-nucleosomes look like 'beads on a string' (Finch et al., 1977). Higher order structures are achieved through the compaction by the linker histone H1.

The DNA-nucleosome polymer chromatin is a highly dynamic macromolecule and exists in different states. Historically, chromatin can be divided into euchromatin, a decondensed or 'open' form with high gene content and transcriptional activity and into heterochromatin, a condensed and gene-poor form (reviewed in Beisel and Paro, 2011).

Chromatin remodeling is ATP-dependent and involves nucleosome sliding, histone exchange, nucleosome eviction and altered nucleosome structures. The accessibility of DNA by the transcriptional machinery is not only dependent on chromatin remodeling, the DNA methylation status and the regulation by noncoding RNAs but also on the modifications of histone tails and the incorporation of histone variants into nucleosomes (reviewed in Lafos and Schubert, 2009).

1.1.2 Histone modifications

The core histones can be subjected to various covalent posttranscriptional modifications such as acetylation, methylation, ubiquitination, phosphorylation, ADP-ribosylation, and sumoylation.

The site of modifications of the core histones are in most cases their N-terminal tails, which protrude from the surface of the nucleosome, thus are exposed to interacting molecules.

The methylation of lysine (K) residues elevates histone hydrophobicity and is likely to alter inter- or intramolecular interactions resulting in a site where 'reader' proteins can bind to the methylated domains (reviewed in Taverna et al., 2007). Depending on the lysine residue and if the respective $\varepsilon\text{-}NH_3^+$ group is mono-, di- or trimethylated the respective methylation mark leads to activation or repression. In plants the methylation of histone H3 lysine 9 (H3K9) and H3K27 is associated with silenced regions whereas the methylation of H3K4 and H3K36 are found in transcribed genes (reviewed in Liu et al., 2010). A methylation of H3K79, found in mammals and yeast, was not detected in *Arabidopsis* (Zhang et al., 2007a), indicating that histone modifications are not a universal code. Histone lysine residues can be mono-, di- or trimethylated in *Arabidopsis*. H3K9me1/2 and H3K27me1 are enriched in constitutive heterochromatin and both H3K9me3 and H3K27me3 are found in euchromatin, but with no significant overlap (Turck et al., 2007). In plants nine predominant chromatin states have been defined, according to their DNA methylation status, GC content, presence of histone variants, and histone modifications (Sequeira-Mendes et al., 2014).

The observations that specific histone modifications are associated with distinct activity states of a gene led to the 'histone code' hypothesis. In this hypothesis it is assumed that (a)

modifications of histone tails are recognized by special chromatin associated 'reader' proteins, which can interpret the modifications and cause gene activation or silencing, and that (b) modifications of the same or different histone tails could be interdependent and lead to various combinations on every single nucleosome (Jenuwein and Allis, 2001). The chromodomain containing DNA methyltransferase protein CHROMOMETHYLASE3 (CMT3) interacts with the H3 tail only when both H3K9 and H3K27 are simultaneously methylated (Lindroth et al., 2004). CMT3 is an example of a reader protein conferring gene repression, and moreover shows that different modifications on the same histone tail are important for recognition by specific reader proteins.

So far there are three ways known to remove histone modifications: 1) removal of modifications by eraser proteins such as histone demethylases (HDMs), 2) replacement of histones by unmodified core histones or variants, and 3) proteolytical cleavage of the N-terminus, which was demonstrated for histone 3 in mice embryonic stem cells (ESC) (Duncan et al., 2008). The *Arabidopsis* Jumonji-domain containing protein RELATIVE OF EARLY FLOWERING6 (REF6) is the first plant demethylase shown to remove methyl groups from H3K27me2 and H3K27me3 (Lu et al., 2011).

Histone methylation is mediated by histone methyltransferases (HMTs), which share a conserved catalytic 130-residue domain, the SET-domain. This domain was named after the three founding members from *Drosophila*: SUPPESSOR OF VARIEGATION3-9 (SU[VAR]3-9), ENHANCER OF ZESTE (E[Z]) and TRITHORAX (TRX) (Tschiersch et al., 1994). The *Drosophila* E[Z] and human E(Z) (or EZH2) SET-domain containing methyltransferases belong to the Polycomb group (PcG) protein family and have been shown to mediate trimethylation of H3K27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). The *Arabidopsis* SET-domain containing protein CURLY LEAF (CLF) is an E[Z] homolog and mediates trimethylation of H3K27 (Saleh et al., 2007; Schmitges et al., 2011; Schubert et al., 2006). The loss of function of CLF leads to ectopic expression of the floral homeotic gene *AGAMOUS* (*AG*) and other genes (Goodrich et al., 1997).

1.2 Polycomb group proteins: writers and readers of histone modifications

The first Polycomb (Pc) mutant was identified in *Drosophila*. Over 60 years ago Pam Lewis observed that *Pc*-mutant male fruit flies, who normally have a thick set of bristles, the so-called sex combs, on their front pair of legs which they use for grasping females during copulation, had additional sex combs on the second and third pairs of legs (Lewis, 1947). This appearance gave the mutant flies the name *Polycomb*.

Polycomb proteins do not act alone but assemble complexes, e.g. in case of E[Z] the presence of the WD40-domain protein ESC (EXTRA SEX COMBS) is necessary for H3K27 methyltransferase activity (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). The so-called POLYCOMB REPRESSIVE COMPLEX2 (PRC2) in *Drosophila* is composed of the four core members: E[Z], ESC, SUPPRESSOR OF ZESTE12 (SU[Z]12) and a second WD40-domain protein P55/NURF-55 (Müller et al., 2002). In knockout mice, for example, the deletion of any of the PRC2 core members leads to embryonic lethality (reviewed in Sauvageau and Sauvageau, 2008).
The PcG-antagonizing SET-domain containing Trithorax group (TrxG) proteins set the transcriptional activation mark H3K4me3, a hallmark of euchromatin, and have been identified in *Drosophila* (Mazo et al., 1990), mammals (Yu et al., 1995) and *Arabidopsis* (Alvarez-Venegas et al., 2003; Mazo et al., 1990; Yu et al., 1995).
The H3K27me3 mark set by the PRC2 complex can be specifically recognized by the POLYCOMB REPRESSIVE COMPLEX1 (PRC1). More precisely, H3K27me3 can be bound by the chromodomain of the PRC1 member POLYCOMB (PC) (Fischle et al., 2003). The PRC1 complex in *Drosophila* is composed of PC, POSTERIOR SEX COMBS (PSC), POLYHOMEOTIC PROXIMAL (PH-p), POLYHOMEOTIC DISTAL (PH-d), dRING1/SCE (SEX COMBS EXTRA) and SEX COMBS ON MIDLEG (SCM), as well as several other proteins like ZESTE or TBP (TATA-binding-protein)-associated factors (Saurin et al., 2001; Shao et al., 1999). It was shown that the *Drosophila* and human PRC1 complexes can modify histones as well. The *Drosophila* PRC1 subunit dRING as well as the human PRC1 members BMI1 (B-cell specific Moloney murine leukaemia virus insertion site1), RING1A and RING1B, were shown to form an E3 ligase complex that monoubiquitylates histone H2A lysine 119 (H2AK119ub) at homeobox (Hox) genes (Cao et al., 2005; Wang et al., 2004). Interestingly, a recent study showed that the *Drosophila* PRC1 can transcriptionally repress target genes also in the absence of H2Aub (Pengelly et al., 2015).
In *Arabidopsis thaliana* both PRC complexes are conserved and will be described in detail below.

1.2.1 Target gene recognition by Polycomb group proteins

In *Drosophila* PcG proteins associate with specific *cis*-regulatory sequences, the Polycomb Response Elements (PREs), necessary for transcriptional repression of Homeobox and PcG target genes (reviewed in Müller and Kassis, 2006). These elements were not (yet) identified in plants, despite *cis*-regulatory sequences were found, for example, at the *AG* locus (Schubert et al., 2006). In plants interaction with transcription factors was shown to target PRC2 and PRC1

to certain loci, but this is unlikely the general mechanisms of PcG recruitment (Liu et al., 2009; Liu et al., 2011b; Lodha et al., 2013; Yang et al., 2013).

In humans over 20% of lncRNAs are bound by PRC2 to guide PRC2 to target genes *in cis* as well as *in trans* (Khalil et al., 2009). Also small ncRNAs (50-200 nucleotides) interact with PRC2 to mediated gene silencing (Kanhere et al., 2010). The role of ncRNAs in plant PRC2 recruitment, however, is not yet clear (reviewed in Heo et al., 2013).

1.2.2 The PRC2 complex in plants

In contrast to *Drosophila* and humans, in *Arabidopsis* at least three different PRC2 complexes with distinct functions, but partially overlapping target genes, have been suggested. The *Arabidopsis* PRC2 is composed of one of three SET domain-containing histone methyltransferases MEDEA (MEA), SWINGER (SWN), and CURLY LEAF (CLF) (Chanvivattana et al., 2004; Goodrich et al., 1997; Grossniklaus et al., 1998; Luo et al., 1999); one of three VEFS domain-containing proteins EMBRYONIC FLOWER 2 (EMF2), FERTILIZATION INDEPENDENT SEED 2 (FIS2), and VERNALIZATION 2 (VRN2) (Gendall et al., 2001; Luo et al., 1999; Yoshida et al., 2001); and the two WD40 domain-containing proteins FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) (Hennig et al., 2003; Ohad et al., 1999). Each PRC2 in *Arabidopsis* contains FIE and MSI1, as well as a histone methyltransferase and a VEFS domain-containing protein (Chanvivattana et al., 2004; Kohler et al., 2003a) (Figure 1. 1).

In *Arabidopsis* PRC2 target genes are covered by H3K27me3, which are about 28% of all annotated *Arabidopsis* protein coding genes (Oh et al., 2008), as well as 43% of all micro RNA genes (Lafos et al., 2011).

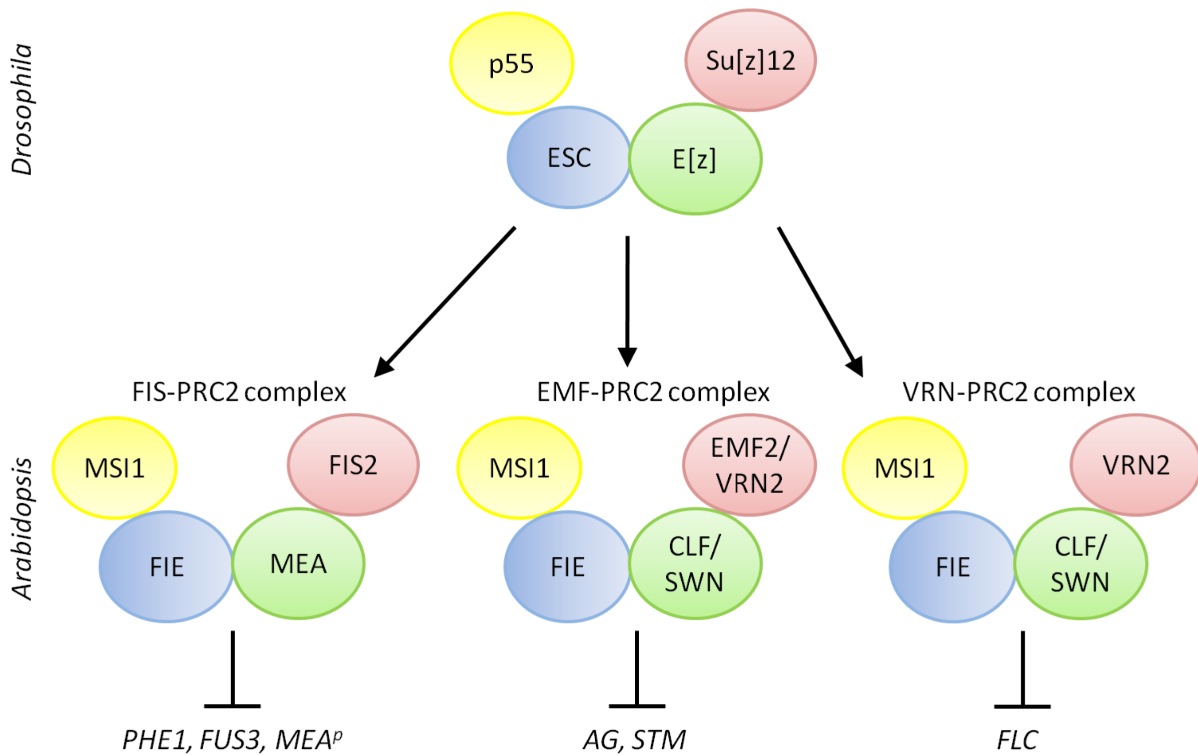

Figure 1. 1: The *Drosophila* PRC2 complex and the three homologous complexes in *A. thaliana*.
All plant PRC2 complexes share the core members MSI1 and FIE, the p55 and ESC homologs from *Drosophila*, respectively. The E[Z] and Su[Z]12 homologs are variable between the plant PRC2 complexes and define their respective target gene specificity. FIS-PRC2 represses *PHE1*, paternal *MEA (MEA^p)*, and *FUS3*, among others (Baroux et al., 2006; Jullien et al., 2006; Makarevich et al., 2006). CLF and SWN also repress *PHE1*, as well as *FUS3* but the involved Su[Z]12 homolog is unclear (Makarevich et al., 2006). FIS2 was also shown to interact with CLF and SWN (Chanvivattana et al., 2004), giving rise to the assumption that there are more PRC2 complexes than have yet been identified. EMF-PRC2 targets *AG* and *STM,* among others. EMF2 and VRN2 share the target genes *AG* and *STM* and were shown to be partially redundant, hence VRN2 is also indicated as Su[Z]12 homolog in the EMF-PRC2. *FLC* repression by VRN-PRC2 requires additional proteins for stable gene repression: together with VRN5, VIN3, and VEL1, VRN-PRC2 forms the so-called PHD-PRC2 complex (De Lucia et al., 2008). Figure was modified from Chanvivattana et al. (2004).

The FIS-PRC2 complex contains FIS2, MEA, FIE and MSI1 and probably other proteins (Kohler et al., 2003a). The genes encoding the members of the FIS-PRC2 are called *FIS* genes, due to their loss-of-function phenotype with autonomous endosperm and seed development in the absence of fertilization (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kohler et al., 2003a; Ohad et al., 1996). A hallmark of *fis*-class mutants is a reduced transmission through the female gamete, but no affected transmission through the male germ line (Chaudhury et al., 1997). The FIS-PRC2 complex regulates the expression of the MADS-box gene *PHERES1* (*PHE1*), which is expressed transiently after fertilization in the embryo and endosperm and whose expression is deregulated in all *fis*-class mutants (Kohler et al., 2003b). *MEA* is expressed in flowers before fertilization and during embryo development and disappears during seed maturation (Grossniklaus et al., 1998; Makarevich et al., 2006), after which the other PRC2 complexes take over to control PcG target gene expression. CLF and SWN were shown to redundantly repress *PHE1* in sporophytic tissue where *MEA* is not expressed (Makarevich et al., 2006). An interesting observation is that in both *clf* and *fie* mutants MEA was derepressed

in sporophytic tissue indicating that a PRC2 complex containing CLF and FIE downregulates *MEA* in vegetative tissues and thus may suppress MEA-FIE complex formation in sporophytes (Katz et al., 2004).

The EMF-PRC2 complex controls the transition from vegetative to inflorescence meristem identity and is a key regulator of flowering time by preventing precocious flowering (Chanvivattana et al., 2004; Yoshida et al., 2001) (Figure 1. 1). EMF-PRC2 members CLF/SWN, EMF2, MSI1 and FIE are ubiquitously expressed during development (Chanvivattana et al., 2004; Goodrich et al., 1997; Hennig et al., 2003; Ohad et al., 1999; Yoshida et al., 2001). CLF and its homolog SWN act partially redundantly (Chanvivattana et al., 2004; Goodrich et al., 1997). *clf* mutants show a pleiotropic phenotype with affected leaf and flower morphology, homeotic transformation of sepals and petals, and early flowering (Goodrich et al., 1997). In contrast, *swn* mutants do not show any abnormalities during their whole life cycle, indicating that these two homologs are not identical in function (Chanvivattana et al., 2004). The *clf swn* mutant, with *clf* and *swn* null-alleles, is only viable when grown in sterile tissue culture, and show a very strong phenotype. When *clf swn* seeds germinate they produce seedlings with narrow, but relatively normal cotyledons, hypocotyls and roots (Chanvivattana et al., 2004). During ageing *clf swn* mutants become abnormal with finger-like projection on cotyledon margins, a shoot apex that cannot initiate leaves but develops into a disorganized mass of green tissue, and in addition a colorless callus-like tissue containing somatic embryos and roots is formed (Chanvivattana et al., 2004).
Like FIS2 and MEA, EMF2 and CLF/SWN directly interact with each other via their VEFS (VRN2/EMF2/FIS2/Su[Z]12) and C5 domain, respectively (Chanvivattana et al., 2004). *emf2* mutants bypass vegetative growth and germinate directly into an inflorescence shoot (Sung et al., 1992). The *emf2* mutant shows a weaker phenotype than the null *clf swn* double mutant (Chanvivattana et al., 2004). The assumption that EMF2 function is masked by partial redundancy with its homolog VRN2 was proven by *emf2 vrn2* double mutant analysis, which resemble *clf swn* mutants (Schubert et al., 2005).
FIE controls shoot and leaf development and low FIE levels produce dramatic morphological aberrations, similar to those of *clf* and *emf2* mutants (Katz et al., 2004).
MSI1, besides being a core component of all three PRC2 complexes, is also a member of the trimeric CHROMATIN ASSEMBLY FACTOR-1 (CAF-1) complex, together with FASCIATA1 (FAS1) and FASCIATA2 (FAS2) (Kaya et al., 2001). CAF-1 guides incorporation of core histones H3 and H4 into chromatin in a replication coupled manner (Kaya et al., 2001). MSI1 is required for the correct temporal and organ-specific expression of

homeotic genes such as *AG*, which is independent from *FAS1* and *FAS2* (Hennig et al., 2003), thus is most likely PRC2-dependent. Recently, it was shown that MSI1 is also part of a histone deacetylase complex that fine-tunes expression of genes responding to the phytohormone abscisic acid (ABA) (Mehdi et al., 2015). Loss of MSI1 led to decreased levels of H3K9ac at several ABA responsive genes in the respective mutant (Mehdi et al., 2015).

The third PRC2 complex in *Arabidopsis* is the VRN-PRC2 (Figure 1. 1), which is involved in the vernalization response. 1918 the German plant physiologist Gustav Gassner first described vernalization, which today is defined as 'the acquisition of the competence to flower by prolonged exposure to cold temperatures in winter annual plants'. Vernalization is necessary to prevent flowering before winter and to promote flowering in spring. Importantly, the vernalized state is mitotically stable but is reset during meiosis (Crevillen et al., 2014).
The MADS-box gene *FLOWERING LOCUS C* (*FLC*) encodes a strong floral repressor which directly represses the floral activator *FLOWERING LOCUS T* (*FT*) ('florigen') (Helliwell et al., 2006). VRN2 is associated with the *FLC* locus independent of temperature and thus presence of the VRN2-PRC2 complex is not sufficient for silencing of *FLC* (De Lucia et al., 2008). Only when VRN2-PRC2 interacts with the PHD (plant homeodomain) finger proteins VRN5 (VERNALIZATION5), VIN3 (VERNALIZATION INSENSITIVE3) and VEL1 (VERNALIZATION5/VIN3-LIKE1), forming the so-called PHD-PRC2, H3K27me3 reaches a level sufficient for silencing (Angel et al., 2011; De Lucia et al., 2008). VRN5 only associates with *FLC* upon low temperature induction of VIN3, ensuring that *FLC* silencing by PHD-PRC2 only occurs during cold (De Lucia et al., 2008). However, the loss of any core component of VRN-PRC2 results in abolished maintenance of de-repression of *FLC* after vernalization (Wood et al., 2006). These observations indicate that VRN-PRC2 is rather important for maintaining the repressed *FLC* state than for initiating it.
Like *swn* also *vrn2* single mutants exhibit no obvious morphological phenotype. VRN2 is not only involved in the vernalization response, indicated by the *vrn2 emf2* double mutant, which shows strongly reduced H3K27me3 levels compared to single mutants (Lafos et al., 2011). A common target of VRN2 and EMF2 is *STM*, which did not comprise altered H3K27me3 levels in *vrn2* and *emf2* single mutants but a significant loss of this mark in the double mutant (Schubert et al., 2006). VRN-PRC2 but not EMF-PRC2 can bind to histone tails carrying H3K4me3 and set the H3K27me3 mark on the same tail (Schmitges et al., 2011). Thus, plants can silence genes carrying activating H3K4me3 by exchanging the Su[Z]12 homolog.

1.2.3 PRC1 proteins in plants

It was long time assumed that animal PRC1 components have no direct homologs in plants, but now evidence for a PRC1 complex in *Arabidopsis* is compelling. The first identified *Arabidopsis* PRC1 protein is the chromodomain-containing protein TERMINAL FLOWER2/LIKE-HETEROCHROMATIN PROTEIN1 (TFL2/LHP1) (Turck et al., 2007). Similar to the *Drosophila* PRC1 subunit POLYCOMB (Pc), TFL2/LHP1 specifically associates with H3K27me3 *in vivo* (Turck et al., 2007; Zhang et al., 2007b). Disruption of the TFL2/LHP1 chromodomain results in loss of H3K27me3 recognition and release of gene silencing (Exner et al., 2009). First, in *tfl2/lhp1* mutants the distribution of H3K27me3 was found to be unaffected (Turck et al., 2007). Recently, two studies showed that H3K27me3 levels are altered in *tfl2/lhp1* mutants (Derkacheva et al., 2013; Wang et al., 2016). Derkacheva et al. (2013) showed that in dividing cells H3K27me3 levels were reduced in *tfl2/lhp1* mutants. The role of TFL2/LHP1 in maintaining H3K27me levels during DNA replication is described below. Wang et al. (2016) revealed that TFL2/LHP1 is important for spreading of H3K27me3 over a locus as the tfl2/lhp1 mutant showed narrow H3K27me3 peaks compared to the wild type.

Arabidopsis AtRING1a and AtRING1b are homologs of the animal PRC1 subunit RING1 (Xu and Shen, 2008). Besides other severe growth defects, the *atring1a atring1b* double mutant shows ectopic meristem formation, complete sterility, and de-repression of embryonic traits, but levels of H3K27me3 are not altered (Chen et al., 2010; Xu and Shen, 2008). AtRING1a was found to physically and genetically interact with AtRING1b as well as TFL2/LHP1 and CLF (Xu and Shen, 2008).

EMBRYONIC FLOWER 1 (EMF1) is the second plant PRC1 component that was identified (Calonje et al., 2008). EMF1 is a plant specific protein which has DNA-binding capacity and is required for floral repression (Aubert et al., 2001; Calonje et al., 2008). *emf1* and the PRC2 mutant *emf2* show the same phenotype (Moon et al., 2003). Like PRC2 mutants, *emf1* mutants show reduced H3K27me3 levels (Calonje et al., 2008) and genome wide EMF1 binding correlated with H3K27me3 (Kim et al., 2012). This led to the assumption that EMF1 is required for maintenance or deposition of H3K27me3. It was long assumed that PRC1 acts downstream of PRC2 but recent data revealed that this is only partially true (see section 1.2.3). EMF1 interacts with MSI1 (Calonje et al., 2008), therefore loss of EMF1 function likely leads to loss of PRC2 recruitment and hence reduced H3K27me3 levels at EMF1 target genes. Although there is no sequence similarity between *Drosophila* PRC1 member PSC and EMF1, both proteins possess similar functions in chromatin compaction and inhibition of chromatin remodeling, which is mediated by a long intrinsically disordered region in both proteins (Beh

et al., 2012). Moreover, EMF1 is important for H2A monoubiquitination as *emf1* mutants show reduced levels of H2Aub (Bratzel et al., 2010).

In mammals the RING-finger proteins BMI1, RING1A and RING1B form an E3 ubiquitin ligase complex that monoubiquitinates H2AK119 (Cao et al., 2005). *Arabidopsis* BMI1 and RING1 homologs are the most recent identified PRC1 component in plants (Sanchez-Pulido et al., 2008). AtBMI1A, AtBMI1B and AtBMI1C were shown to interact with both AtRING1A and AtRING1B, as well as TFL2/LHP1 and EMF1*in vitro* (Bratzel et al., 2010; Bratzel et al., 2012; Chen et al., 2010). AtRING1A can bind itself and directly interacts with AtRING1B, TFL2/LHP1, EMF1 and the PRC2 component CLF (Bratzel et al., 2010; Xu and Shen, 2008). Both, the *atring1a/b* and *atbmi1a/b* double mutants show a de-repression of embryonic traits in somatic cells and de-repression of key stem cell regulatory genes in both apical meristems, revealing that AtRING1 and AtBMI1 proteins have non-redundant functions in post-embryonic plant development (Bratzel et al., 2010; Chen et al., 2010). Bratzel et al. (2010); (2012) showed that AtBMI1A, AtBMI1B, AtBMI1C, AtRING1A and AtRING1B can monoubiquitinate H2A *in vitro*, hence revealing the conservation of PRC1 function in animals and plants. Importantly, *atbmi1a/b/c* triple mutants completely lost the H2Aub mark, revealing that AtBMI1s are important for *in vivo* H2A monoubiquitination activity (Yang et al., 2013). It was also shown that deposition of H2Aub at seed maturation genes depends on AtBMI1 and VAL (*VP1/ABI3-LIKE*) proteins, hence indicating that, like PRC2, also PRC1 requires interaction partners for proper silencing of certain target genes (Yang et al., 2013).

These results indicate that in plants seven PRC1 components, namely TFL2/LHP1, EMF1, AtRING1A, AtRING1B, AtBMI1A, AtBMI1B, and AtBMI1C, are conserved. The composition of the plant PRC1 is not yet clear but, like in mammals and *Drosophila*, presence of several PRC1 complexes, which repress genes by H2Aub-dependent and -independent mechanisms, is indicated (reviewed in Merini and Calonje, 2015).

1.2.3 Polycomb targeting in plants: PRC1 takes the lead

According to molecular and functional evidence, it was long believed that targeting of PRC1 and PRC2 in *Drosophila* follows a hierarchical order: First PRC2 sets H3K27me3, which is then recognized and bound by the PRC1 subunit Pc, hence recruiting PRC1 to H3K27me3 containing target genes. PRC1 would then mediate H2A monoubiquitination, leading to chromatin compaction and stable gene repression. This hierarchical recruitment model was extended to plants. A recent study in plants revealed that this model is not correct (Yang et al., 2013) and was later also shown to be the case in vertebrates (Blackledge et al., 2014; Cooper et al., 2014; Kalb et al., 2014). Yang et al. (2013) showed that AtBMI1s are required for initial

repression of embryonic traits, because these mutants failed to initiate vegetative development, whereas *clf/swn* double mutants could undergo phase transition and then showed progressive loss of cell differentiation. Consistently, H3K27me3 levels were reduced at seed maturation genes in *atbmi1a/b/c* mutants, indicating that PRC1 recruits PRC2 to certain target genes (Yang et al., 2013). Importantly, stem cell maintenance genes and floral organ identity genes showed increased levels of H3K27me3 in *atbmi1a/b/c* (Yang et al., 2013). Conversely, *clf/swn* mutants showed increased levels of H2Aub, indicating that PRC1 and PRC2 directly or indirectly regulate each other´s activity, presumably to maintain a balance of repressive marks (Yang et al., 2013). Although other studies now showed that PRC2 is recruited to certain PcG target genes in a PRC1 dependent manner, targeting of PRC1 and PRC2 is also independent of each other (reviewed in Merini and Calonje, 2015). For example, PRC2 can be recruited to certain target genes via interaction with transcription factors such as the ASYMMETRIC LEAVES (AS) complex or AG (Liu et al., 2011b; Lodha et al., 2013). Also RNA based or PRE-like motif dependent recruitment of PRC2 are likely to occur in plants (reviewed in Heo et al., 2013; Mozgova et al., 2015). Additionally, VRN-PRC2 can bind to histone tails carrying H3K4me3 and set the H3K27me3 mark on the same tail *in vitro* (Schmitges et al., 2011), but it is unclear if and how this occurs *in planta*. PRC1 recruitment was also shown to depend on transcription factors, lncRNAs and PRE-like elements in plants (Ariel et al., 2014; Hecker et al., 2015; Latrasse et al., 2011; Liu et al., 2009; Yang et al., 2013). During germination PRC1 was shown to be recruited to H3K4me3 at active seed developmental genes by interaction with ALFIN1-like proteins (Molitor et al., 2014). PRC1 then induces a state switch of these genes and recruits PRC2 to deposit H3K27me3 for stable gene silencing during vegetative development (Molitor et al., 2014). Interestingly, propagation of H3K27me3 during DNA replication was shown to depend on LHP1-mediated PRC2 recruitment and will be discussed below.

1.2.4 Propagation of repressive marks

Mitosis poses a problem for maintaining the repressed state of a gene: semi-conservative DNA replication, during which unmodified histone proteins are incorporated into the newly formed nucleosomes, can dilute histone marks such as H3K27me3. Especially for small miRNA genes the coverage by H3K27me3 can be affected when more and more unmodified nucleosomes are incorporated during successive rounds of mitosis. Recently, Derkacheva et al. (2013) revealed that LHP1 interacts with the PRC2 component MSI1 for confidential inheritance of H3K27me3 to both daughter strands during DNA replication. LHP1 remains bound to parental H3K27me3 during DNA replication and recruits PRC2 via interaction with MSI1 to nucleosomes which contain H3K27me3; PRC2 can then trimethylate H3K27 on neighboring, newly incorporated

histones to sustain coverage of this repressive mark on both daughter stands (Derkacheva et al., 2013).

CLF is not present in nuclei during mitosis (Schubert et al., 2006). Most likely its expression is regulated post-translationally via the F-box component of an E3-ligase, UPWARD CURLY LEAF1 (UCL1), which targets CLF for degradation in the ubiquitin-26S-proteasome pathway (Jeong et al., 2011). The *tfl2/lhp1* and *clf* mutants showed narrow H3K27me3 peaks at PcG target genes (Wang et al., 2016), which could be explained by coordinated interaction of LHP1 and CLF to maintain broad coverage of H3K27me3 after DNA replication. However, it remains unclear whether the model proposed by Derkacheva et al. (2013) is the only mechanism or is the case for all PRC2 target genes throughout development, because the *tfl2*/*lhp* mutant shows a mild phenotype (Turck et al., 2007). The *Drosophila* PRC2 was shown to target dense chromatin to deposit H3K27me3 independent of PRC1 to maintain, but not to initiate gene repression (Yuan et al., 2012). This mechanism could also play a role in maintenance of H3K27me3 levels after mitotic divisions in plants.

1.3 The *Arabidopsis* protein BLISTER is linked to PRC2

In order to identify new members and interaction partners of the plant PRC2, CLF was used as 'bait' in a yeast-two-hybrid screen. In this screen the plant specific protein BLISTER (BLI) was identified. BLI is a 714 amino acid long protein, encoded by a single copy gene consisting of 13 exons, and has homologs in monocots as well as dicots but not in the animal kingdom (Schatlowski et al., 2010). The *in planta* interaction domains of BLI and the PRC2 methyltransferase CLF were determined to be the BLI SMC (STRUCTURAL MAINTENANCE OF CHROMOSOMES) and the CLF CXC (cysteine rich pre-SET) domain (Schatlowski et al., 2010). In plants and other eukaryotes SMC proteins are essential for chromosome condensation, DNA repair and recombination, sister chromatid cohesion, and play a role in transcription (reviewed in Schubert, 2009). SMC proteins are long coiled coils with an ATPase domain on the one, and a hinge domain at the other end of the protein, with the latter being a linker domain for SMC protein complex formation (Schubert, 2009). Importantly, unlike other SMC domain proteins, BLI contains no hinge or ATPase domain and thus most likely fulfills other functions in the plant (Schatlowski et al., 2010). Moreover, the BLI SMC domain shows similarities to bacterial SMC domains but not to eukaryotic ones. In yeast-two-hybrid interaction studies BLI was furthermore shown to directly interact with VRN2 and SWN, but not with EMF2, MSI1 or TFL2/LHP1 (Schatlowski, 2010).

1.3.1 The *bli-1* phenotype

BLI is expressed in many tissues and developmental stages, ranging from seedlings to siliques (Schatlowski et al., 2010). *bli-1* is a T-DNA insertion mutant with a pleiotropic phenotype affecting seed, leaf, and flower development (Schatlowski et al., 2010). The transcript level of *BLI* is severely reduced in the *bli-1* mutant, indicating that *bli-1* is a null or severe knockdown allele (Schatlowski et al., 2010). *bli-1* mutants show ectopic outgrowth of cells from cotyledons, true leaves, stems, flowers, and siliques, which resemble blisters (Figure 1. 2 B-C).

Figure 1. 2: *bli* mutant phenotypes.
A) Phenotype of adult, strong *bli* mutants, *bli-1* and *bli-11* (characterized in this study), in comparison to weak *bli* mutants, *bli-2* and *bli-3,* and the wild type Col-0. The newly-characterized *bli-11* mutant resembles *bli-1*. Both mutants show a strong, pleiotropic phenotype. The weak *bli* mutants, *bli-2* and *bli-3,* resemble the wild type. B-C) Blister-like structures at cotyledons of a *bli-1* seedling (B) and a flower of *bli-11* (C). Arrowheads point to blister-like structures in B) and C).

1.3.2 BLI molecular function

In *bli-1* the H3K27me3 target genes *PISTILLATA (PI), SEPALLATA2 (SEP2)* and *SEP3* were significantly up-regulated whereas others, e.g. *AG, FT,* showed no mis-regulation (Schatlowski et al., 2010). The H3K27me3 levels at *FT, AG, SEP2,* and *SEP3* were not altered in *bli-1* seedlings, indicating that BLI is not required for H3K27me3 deposition or maintenance at these genes under the tested conditions (Schatlowski et al., 2010). BLI was identified as an important regulator of cold-stress responses (Purdy et al., 2010). In their study, Purdy et al. (2010) showed that cold-responsive genes were not properly induced in the weak *bli-2* and *bli-3* mutants (Figure 1), revealing that these knockdown mutants, despite morphologically resembling the wild type, display intrinsic gene regulatory defects. The observation that BLI acts in cold responses might indicate an interaction of BLI with a vernalization related PRC2 complex containing CLF and VRN2. The analysis of BLI subcellular localization was carried out using BLI-GFP (GREEN FLUORESCENT PROTEIN) fusion proteins. In *N. benthamiana* leaf epidermis cells a *35S:cBLI-GFP* construct localized to nuclei and cytoplasmic speckles

(Schatlowski et al., 2010). In *Arabidopsis* root cells *35S:BLI-GFP*, containing C-terminally truncated *BLI*, localized to nuclei and cytoplasmic speckles as well (Purdy et al., 2010).

1.3 Stress responses in plants

Abiotic and biotic stress were shown to affect the chromatin state in plants: histone modifications were induced or removed in plants that were subjected to specific forms of stress such as cold, drought, or pathogen infection (Ding et al., 2012; Jaskiewicz et al., 2011; Kwon et al., 2009; Liu et al., 2014; Sani et al., 2013). So far, it could not conclusively be resolved if these modifications are epigenetic modifications, hence are mitotically or meiotically heritable, or if they are transient chromatin modifications which are not heritable and therefore not epigenetic. The role of Polycomb or Trithorax proteins in stress responses is just emerging. PRC1, PRC2 and TrxG proteins were shown to directly bind stress-responsive genes and loss of PcG and TrxG protein function altered the plant´s stress tolerance (Alexandre et al., 2009; Han et al., 2012; Kim et al., 2010; Qin et al., 2008). Additionally, some PcG proteins probably also regulate stress responses in a PcG-independent manner, e.g. PRC1 members were shown to target a drought-responsive TF to 26S-proteasome-mediated proteolysis (Qin et al., 2008). The role of PcG and TrxG proteins in the control of stress responses in plants is summarized in the following article (Kleinmanns and Schubert, 2014).

1.3.1 Polycomb and Trithorax group protein-mediated control of stress responses in plants (Kleinmanns and Schubert, 2014)

DE GRUYTER Biol. Chem. 2014; 395(11): 1291–1300

Review

Julia Anna Kleinmanns and Daniel Schubert*

Polycomb and Trithorax group protein-mediated control of stress responses in plants

Abstract: A plant's experience of abiotic or biotic stress can lead to stress memory in order to react faster and more efficiently to subsequent stresses. Molecularly, the memory of a stress can rely on stable inheritance through mitotic and meiotic cell divisions, thus epigenetic inheritance. The key epigenetic regulators are DNA cytosine methyltransferases and the Polycomb group (PcG) and Trithorax group (TrxG) proteins, which control numerous developmental processes. PcG and TrxG proteins act antagonistically on stable gene repression through mediating trimethylation of histone H3 lysine 27 (H3K27me3) and H3K4me3, respectively, and target thousands of genes in plants, including many genes responsive to stress. The role of PcG/TrxG proteins in regulating stress responses and memory, however, is just emerging. While it is well investigated that stress can induce changes of histone modifications at genes regulated by stress, it is largely unclear whether these changes are mitotically and/or meiotically heritable, hence confer somatic and/or transgenerational stress memory. As the literature on the role of DNA methylation in regulating stress responses has recently been extensively summarized, we focus this review on the current knowledge on the role of PcG and TrxG in stress responses and memory.

Keywords: biotic and abiotic stress; epigenetics; H3K4me3; H3K27me3; priming; somatic and transgenerational memory.

DOI 10.1515/hsz-2014-0197
Received May 15, 2014; accepted July 28, 2014; previously published online July 31, 2014

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Introduction – stress responses and memory

During their life cycle, plants can encounter a large variety of stresses: unfavorable soil composition, extreme weather conditions such as drought, heat or water logging, herbivore or pathogen attacks, air pollution, wounding, a lack of symbionts, etc. The plants' sessile lifestyle requires a rapid response to stress, e.g., by altered metabolite production and gene expression. If a stress is experienced several times during the plant's life cycle, the plant may 'remember' the first encounter with a (mild) stress to cope with subsequent (stronger) stresses more successfully. The plant's response to a second stress is then faster and more effective, a phenomenon already described in the early 1930s (Chester, 1933) and today referred to as 'priming'. Priming is a way of making a plant 'ready for battle' and can be induced when an organ is challenged by a pathogen or microbe attack, by beneficial microbes, by wounding, by treatment with natural or synthetic molecules but also by abiotic stresses such as salt or drought stress (reviewed in Conrath, 2011). Several cases have been described where local experience of a stress results in systemic spreading of the information, inducing the primed state in cells that were not exposed to the stress (Kohler et al., 2002). Importantly, after pathogen-induced priming, gene expression and metabolism are changed in the primed cells but a profound defense response is not established. The faster and stronger response of primed cells toward subsequent abiotic and biotic stresses is dependent on 'signal amplifiers' in the form of mitogen-activated protein kinases (MPK) 3 and 6 (Beckers et al., 2009). The authors showed that expression of *MPK3* and *MPK6* was induced by priming; however, the resulting proteins remained inactive under pre-stress conditions. Upon stress, MPK3 and MPK6 may be activated through the MPK signaling cascade involving MPK kinases (MPKKs) and MPKK kinases (MPKKKs), leading to an enhanced expression of defense genes such as *pathogenesis-related protein* 1 (*PR1*) (Beckers et al., 2009). In addition, priming

can prepare or 'train' genes for enhanced expression after a second stress, which differentiates these genes from 'non-trainable' genes that are induced to a similar expression level after each stress treatment (Ding et al., 2012). In contrast to priming, which is not accompanied by a profound stress response, the process of systemic acquired resistance (SAR) is usually a result of a strong defense response in a primarily infected organ. When an organ is challenged by a pathogen attack, a mobile signal is generated in the primarily infected organ and is rapidly transported to uninfected organs. This signaling cascade involves the non-proteinaceous amino acid pipecolic acid (Navarova et al., 2012) and the plant hormone salicylic acid, among others, and leads to induction of defense-related proteins such as PR1 or transcription factors (TFs) such as WRKY family TFs (reviewed in Kachroo and Robin, 2013). Interestingly, the induction of SAR in one organ can also lead to priming of uninfected organs, thereby ensuring a rapid and more effective response upon a subsequent stress in these organs (Conrath et al., 2006; Jaskiewicz et al., 2011). The primary defense response during SAR is energy consuming and can lead to a trade-off in regard of seed production and growth (van Hulten et al., 2006), especially if the plant has to compete for resources with plants that do not invest energy into defense responses. The induction of a primed state, on the other hand, is a cost-efficient way to protect against biotic stress, because in the absence of pathogens growth rate and seed production are only mildly reduced (van Hulten et al., 2006). The experience of a stress and subsequent induction of priming, SAR, or related immune responses are thus beneficial in environments with a high risk of repeated stress exposure and may be more relevant in long-lived perennial in contrast to annual plants. In addition, the memory of a stress is likely not only important for the plant itself but also for its offspring; therefore, it could have adaptive value, especially when the offspring will grow in a similar environment and in similar conditions as the mother plant.

Mechanisms of somatic and transgenerational plant stress memory

As plants lack a nervous system, memory of a stress must be conferred by different means. A given stress can be memorized locally, so that the cell/tissue exposed to the priming stress shows a potentiated response to a second stress. The information can also spread systemically to already existing organs as observed in SAR. In addition, newly developing cells and organs and even the offspring may acquire a potentiated stress response (somatic and transgenerational stress memory, respectively). At least three distinct mechanisms of somatic and transgenerational stress memory are conceivable and have been partially observed: (i) generation and inheritance of stress-induced proteins, RNAs, or metabolites ('memory compounds') that directly interfere with stress responses; (ii) epigenetic changes that prime the chromatin of newly developing cells and/or the offspring to allow fast transcriptional responses upon stress exposure; and (iii) changes in the DNA sequence by mutations that alter gene expression (Figure 1). In addition, signaling from the mother plant to the developing embryo may increase stress tolerance in the next generation (Figure 1) or the developing embryo itself may be exposed to the stress. However, both cannot be considered as transgenerational inheritance phenomena (reviewed in Heard and Martienssen, 2014). To unequivocally detect transgenerational epigenetic inheritance, careful setup of experimental conditions is required, which ideally requires a stress-free generation between the generation exposed to a priming stress and the generation experiencing the second stress.

Which mechanisms are the most likely to confer somatic and transgenerational stress memory? The memory in the form of molecules would be 'diluted' by successive rounds of cell division and during gamete formation, embryogenesis, and seedling development, thus is a rather unlikely mechanism to confer long-term memory. Especially if the priming stress is experienced early during development, this dilution process would lead to a loss of memory (self-perpetuation of memory compounds is considered to be an epigenetic process). Mutations of the DNA are unlikely to play a role in stress memory, as they are not directional and not reversible. Moreover, mutations in the DNA may lead to a trade-off if a plant shows a constitutive and costly stress response (Bowling et al., 1994). Additionally, the observation that non-stressed plants do not show constitutive expression of defense or stress-responsive genes such as PR1 (van Hulten et al., 2006) suggests that DNA mutations are rather unlikely to contribute to somatic and transgenerational memory. Hence, the most likely form of somatic and transgenerational memory is the storage of the stress memory through epigenetic mechanisms.

Epigenetic mechanisms are important for regulation of gene expression and genome integrity. The term 'epigenetic' defines mitotically and/or meiotically heritable, potentially reversible modifications of chromatin and (changes in) its structure that are not caused by DNA

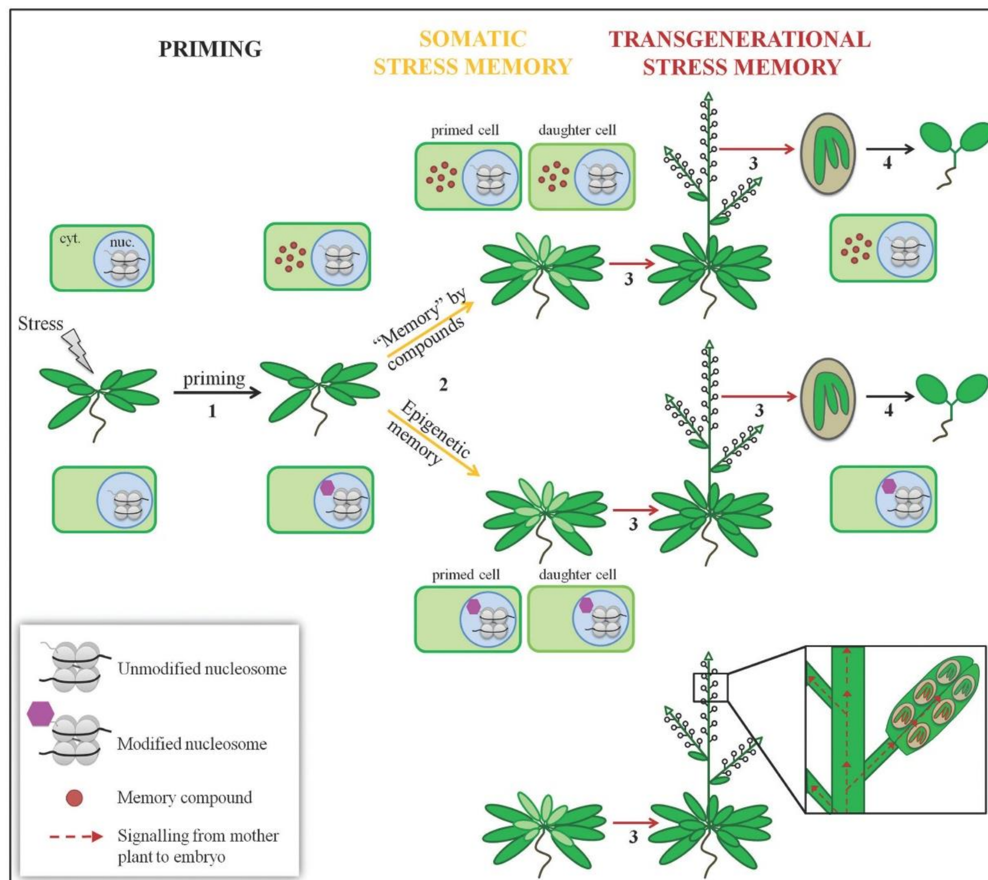

Figure 1 Model of somatic and transgenerational stress memory of plants.
(1) When a plant is exposed to (a mild) stress, priming can occur, leading to faster and stronger responses to a second stress. (2) This primed state is maintained or memorized by the plant either through 'memory compounds', e.g., proteins such as inactive MAP kinases (Beckers et al., 2009), RNAs, or metabolites that were generated or modified in response to stress/priming, or through epigenetic mechanisms such as histone lysine or DNA methylation. We define 'somatic stress memory' as a stress memory that is maintained during successive rounds of cell division, thus also newly formed organs will comprise memory of a given stress (light green leaves). While stress memory in non-dividing cells and SAR rely at least partially on memory compounds, they are unlikely to play a major role in somatic stress memory as they will be diluted through successive cell divisions (unless they would be self-perpetuating and therefore epigenetic). (3) Stress memory may be transmitted through the germ cells to the next generation, through epigenetic mechanisms, transmission of memory compounds, or signaling between the stress-exposed mother and the developing embryo. Hence, the embryo will comprise a memory of the stress that was experienced by the mother plant. (4) The embryo will develop into a seedling and may maintain the stress memory during somatic cell divisions and organogenesis. cyt., cytoplasm; nuc., nucleus.

mutations (Russo et al., 1996). Chromatin refers to DNA and all associated proteins, such as histones or TFs, and includes all linked (nascent) RNAs. Importantly, according to the definition by Russo et al. (1996), chromatin modifications are only epigenetic if they are inherited through mitosis and/or meiosis independently of the stimulus. We will therefore differentiate between 'chromatin marks', defined as short-term modifications of chromatin in response to priming and stress, and 'epigenetic marks', defining modifications of chromatin that are stable during somatic cell division, gamete formation, and that may persist in the offspring to confer stress resistance.

Nucleosomes, as a basic repeating unit of chromatin, consist of a histone octamer, composed of two copies each of histone H2A, H2B, H3, and H4 around which about 147 bp of DNA are wrapped (Luger et al., 1997). Histones can be post-translationally modified by diverse chemical groups, leading to altered activity of the associated genes. DNA can be methylated at cytosine residues (5-methylcytosine), a modification important for gene regulation and genome integrity (reviewed by Zhang et al., 2010).

DNA methylation and methylation of certain histone residues, including H3 lysine 27 trimethylation (H3K27me3), are considered as epigenetic marks when they confer stable repression or activation of genes throughout development and cell divisions. As the role of DNA methylation in plant stress responses was recently reviewed elsewhere (Sahu et al., 2013), we will focus this review on the other major epigenetic system, the Polycomb group (PcG)/Trithorax group (TrxG) proteins, and their role in abiotic and biotic stress responses and somatic and transgenerational memory. Interestingly, while the role of PcG/TrxG in regulating stress responses and memory is just emerging, genome-wide analyses of the PcG- and TrxG-mediated histone modifications revealed a large fraction of stress-responsive genes among the PcG/TrxG target genes (Zhang et al., 2007; Oh et al., 2008), suggesting an important function in regulating stress-responsive genes.

The role of PcG and TrxG proteins in stress responses

PcG proteins act in several, so-called Polycomb repressive complexes (PRC). The highly conserved PRC2 mediates H3K27me3, a modification associated with gene repression. In *Arabidopsis*, PRC2 is composed of one of three SET domain-containing histone methyltransferases MEDEA (MEA), SWINGER (SWN), and CURLY LEAF (CLF); one of three VEFS domain-containing proteins EMBRYONIC FLOWER 2 (EMF2), FERTILIZATION INDEPENDENT SEED 2 (FIS2), and VERNALIZATION 2 (VRN2); and two WD40 domain-containing proteins [FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1)] (reviewed in Hennig and Derkacheva, 2009). PRC1 represses genes through mono-ubiquitination of H2A (H2Aub) and chromatin remodeling. In *Arabidopsis*, at least seven PRC1 proteins were identified: AtRING1a, AtRING1b, AtBMIa (B cell-specific Moloney murine leukemia virus integration site 1), AtBMI1b, AtBMI1c, EMBRYONIC FLOWER 1 (EMF1), and LIKE-HETEROCHROMATIN PROTEIN 1 (LHP1) (reviewed in Calonje, 2014). The TrxG proteins, such as ARABIDOPSIS TRITHORAX1 (ATX1) (Alvarez-Venegas et al., 2003), ULTRAPETALA1 (ULT1) (Carles and Fletcher, 2009), and BRAHMA (BRM) (Farrona et al., 2004), act antagonistically to PRC2 by activating gene expression through setting H3K4me3 and by ATP-dependent chromatin remodeling.

A first function for PRC2 in regulating stress responses was revealed with the identification of the PRC2 subunit MSI1 as a negative regulator of drought stress responses in *Arabidopsis* (Alexandre et al., 2009). Analyzing a *MSI1* co-suppression line (*msi1-cs*), Alexandre et al. (2009) revealed that reduced expression of *MSI1* increased drought stress tolerance compared with the wild type. Moreover, genes containing abscisic acid (ABA) response elements were upregulated in the *msi1-cs* line, whereas genes involved in ABA synthesis or signaling were not affected (Alexandre et al., 2009). ABA is a plant hormone induced by abiotic stress and, among others, important for stomatal closure upon drought stress (reviewed by Tuteja, 2007). MSI1 is part of the PRC2 but also a subunit of the chromatin remodeling complex CAF1 (Kaya et al., 2001). As the level of the PcG mark H3K27me3 was not analyzed in *msi1-cs* plants, it remains unclear whether the PcG function of MSI1 is responsible for the observed phenotype. A study in barley revealed that the expression of the PRC2 components *HvE(Z)* (*Enhancer of zeste*, a *CLF* homolog) and *HvFIE* were increased by ABA treatment (Kapazoglou et al., 2010). Also, loss of the CLF interacting protein BLISTER (BLI) (Schatlowski et al., 2010) leads to misregulation of ABA-responsive genes in the corresponding mutant (our unpublished data). Moreover, *BLI* was shown to be required for cold resistance and expression of cold-responsive genes such as *COR15A* (*COLD REGULATED15A*) (Purdy et al., 2010).

The PRC1 components BMI1a and BMI1b are also known as DREB2A-INTERACTING PROTEIN 2 (DRIP2) and DRIP1, respectively, and are important negative regulators of drought-responsive gene expression by targeting the TF DREB2A to 26S proteasome-mediated proteolysis (Qin et al., 2008). However, whether BMI1a/b also repress drought-responsive genes in a PcG-dependent manner was not resolved in this study.

Loss of function of the TrxG gene *BRM* leads to increased hypersensitivity to ABA and increased drought tolerance (Han et al., 2012). Binding of BRM to the drought stress/ABA-inducible TF gene *ABA INSENSITIVE5* (*ABI5*) suggests that BRM may be directly recruited to stress-responsive genes (Han et al., 2012). Importantly, even in the absence of drought stress/ABA, BRM represses *ABI5* expression, thus preventing a constitutive drought stress response (Han et al., 2012).

The *Arabidopsis* PcG proteins EMF1 and EMF2 repress several categories of stress-induced genes such as cold stress-induced *COR15A* (Kim et al., 2010). Among others, under non-stress conditions the PRC1 protein EMF1 directly binds to genes involved in biotic and abiotic stress, and these binding sites largely overlap with H3K27me3 sites (Kim et al., 2012). Kwon et al. (2009) revealed that during cold temperatures in *Arabidopsis*, the occupancy of H3K27me3 at *COR15A* and another cold responsive

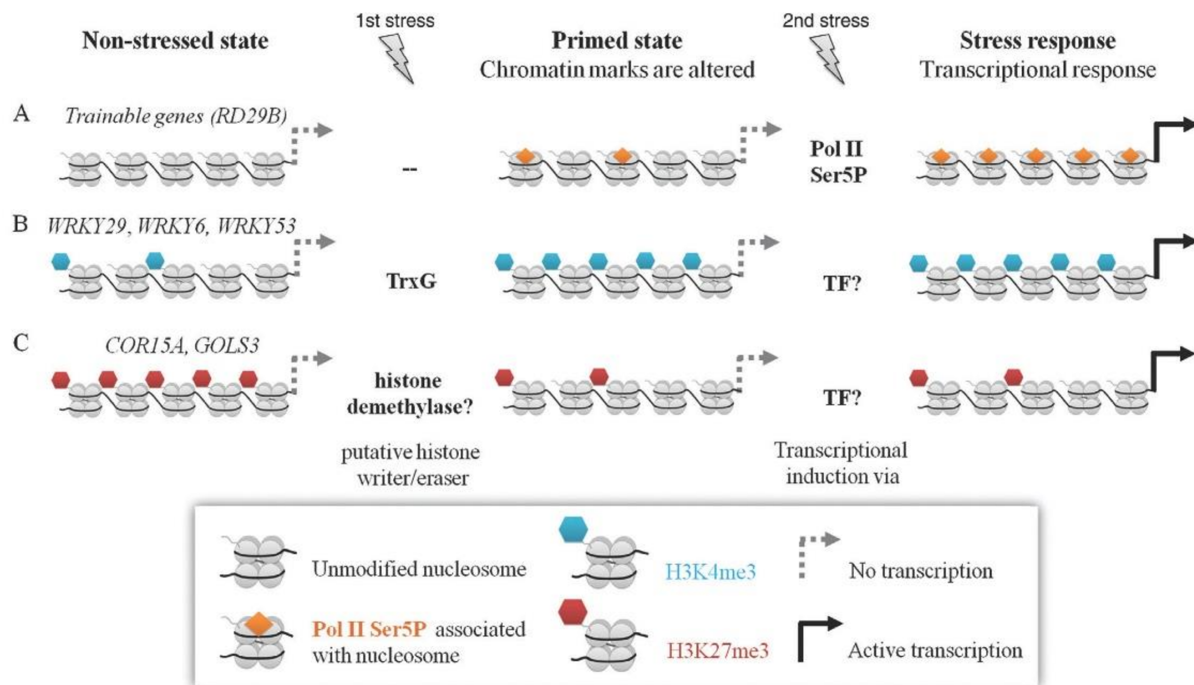

Figure 2 Alteration of chromatin marks at stress-responsive genes during stress.
The experience of a first stress stimulus leads to alteration, e.g., writing or removal, of chromatin marks. These alterations may be sustained during consecutive cell divisions. Upon exposure to a repeated stress, chromatin modifications prime genes for activation. Altered chromatin marks after priming do not necessarily need to affect gene expression. (A) Trainable genes, such as *RD29B*, gain Pol II Ser5P (Pol II phosphorylated at serine 5) during priming but are not transcribed in the recovery phase (Ding et al., 2012). After a second stress, Pol II Ser5P increases at trainable genes, thus increasing transcriptional induction of these genes. (B) Genes with little or no H3K4me3 coverage are not expressed under non-stress conditions. The priming event will increase or set the H3K4me3 mark and poise these genes for activation in case a second stress is experienced (e.g., *WRKY29*, *WRKY6*, and *WRKY53*) (Jaskiewicz et al., 2011). H3K4me3 is likely conferred by TrxG proteins. Stress-inducible or -activated TFs may only be recruited to H3K4me3-primed genes for induction of gene expression. (C) Loss of H3K27me3 during priming at *COR15A* and *GOLS3* is probably an active process requiring histone lysine demethylases (Kwon et al., 2009). Stress-responsive genes retaining reduced H3K27me3 occupancy during recovery may require the activity of TFs for induction, which can only access H3K27me3-depleted genes.

PcG target gene, *GOLS3* (*GALACTINOL SYNTHASE3*), decreased gradually, while transcription was quickly induced (Kwon et al., 2009). Upon return to ambient temperatures, the cold-induced decrease in H3K27me3 levels at *COR15A* and *GOLS3* was sustained, whereas transcription was repressed again (Kwon et al., 2009) (see Figure 2). Therefore, H3K27me3 occupancy at these genes does not inhibit transcription upon cold stress; however, activation of *COR15A* and *GOLS3* leads to H3K27me3 removal (Kwon et al., 2009). Re-exposure of cold-treated plants to cold did not increase transcriptional induction of *COR15A* and *GOLS3*, despite maintenance of reduced H3K27me3 levels (Kwon et al., 2009). Taken together, the studies by Kim et al. (2010, 2012) and Kwon et al. (2009) revealed that (i) PcG proteins bind to stress-responsive genes and that (ii) H3K27me3 occupancy at these genes changes upon stress (Kwon et al., 2009; Kim et al., 2010, 2012).

Training genes for enhanced stress resistance

Stress-responsive genes can be divided into two distinct groups, according to their ability to be primed by a first stress encounter. Ding et al. (2012) showed that cold and drought stress-inducible *COR15A* belongs to the group of so-called non-trainable genes, so that the expression levels of the genes increase during stress, revert to a non-transcribed state after the stress, and reach a similar expression level during a subsequent stress as during the first stress (Ding et al., 2012). 'Trainable' genes, however, such as *RESPONSIVE TO DESSICATION 29B* (*RD29B*), show a 'transcriptional memory', so that the induction of these genes is much higher during the second stress compared with the first one, while remaining non-transcribed

during the recovery phase in between each stress (Ding et al., 2012). To further analyze the mechanism of transcriptional memory during and after consecutive drought stress treatments, the authors analyzed the TrxG-mediated H3K4me3 levels at non-trainable vs. trainable genes, as well as the occupancy with Pol II Ser5P (RNA polymerase II phosphorylated at serine 5 of the C-terminal domain), a Pol II modification associated with transcription initiation and early elongation (reviewed in Hajheidari et al., 2013). At non-trainable genes, Ding et al. (2012) found increased levels of both H3K4me3 and Pol II Ser5P only during the stress treatments, whereas during recovery phases H3K4me3 and Pol II Ser5P levels decreased again to pre-stress levels (see Figure 2). During each stress treatment, the non-trainable genes showed the same level for H3K4me3 and Pol II Ser5P, respectively, consistent with the fact that the transcription of non-trainable genes reached the same level during each stress treatment (Ding et al., 2012). At trainable genes, levels of both H3K4me3 and Pol II Ser5P increased during the first stress treatment, but did not revert to pre-stress levels during recovery phases. Moreover, these modifications increased even further during subsequent stress (Ding et al., 2012). In plants mutant for the H3K4 trimethylase *ATX1*, transcription and H3K4me3 levels at both trainable and non-trainable genes were strongly reduced during stress treatments (Ding et al., 2012). However, potentiated expression of trainable genes was still occurring in *atx1* mutants, suggesting that ATX1 is not required for the memory but for high-level H3K4me3. In addition, H3K4me3 and Pol II Ser5P levels at trainable genes were lost at 7 days after the last stress treatment in *Arabidopsis* wild type, hence suggesting that no long-term, i.e., heritable, somatic memory was established (Ding et al., 2012). H3K27me3 occupancy at all tested trainable genes did not change substantially during consecutive drought stress treatments and recovery phases, although these genes were transcriptionally active during the stress phases (Liu et al., 2014). This observation reinforces the results by Kwon and colleagues (2009) that despite being covered with H3K27me3, genes can become transcriptionally active during stress. Distinct trainable genes appear to be differently affected by loss of the H3K27 trimethylase *CLF*: one subset of the tested trainable genes in *clf* mutants showed decreased H3K27me3 levels, while expression upon stress was increased and this increase was even maintained during recovery (Liu et al., 2014). The other subset showed no alteration in H3K27me3 levels but reduced transcript levels during stress (Liu et al., 2014). Surprisingly, loss of *CLF* resulted in a higher sensitivity to drought stress while *msi1-cs* lines have a higher resistance (Alexandre et al., 2009; Liu et al., 2014), suggesting that different PRC2 members have distinct functions in regulating stress responses or that the role of MSI1 in drought stress regulation is due to its function in additional complexes. Higher sensitivity of *clf* mutants may be caused by changes in ABA levels, which were decreased to 40–60% of wild-type levels (Liu et al., 2014). Thus, *CLF* has an important function in the regulation of stress-responsive genes (Liu et al., 2014); however, whether these effects rely on direct CLF binding to target genes remains unclear.

Epigenetic mechanisms of priming and somatic memory

The above-mentioned studies on the role of PcG and TrxG proteins in stress responses did not focus on long-term or transgenerational memory of stress. To analyze short- and long-term memory and potentially reveal epigenetic inheritance of stress memory, chromatin modifications need to be analyzed in tissues that had not been exposed to the stress, either in the same or in subsequent generations. Several elegant studies have been published in recent years, which either analyzed histone modifications (i) immediately or a few days after priming of plants, addressing somatic stress memory, or (ii) in consecutive generations that were exposed to priming/stress, to address transgenerational stress memory.

To analyze PcG- and TrxG-mediated somatic stress memory, a recent study analyzed genome-wide changes of H3K27me3 and H3K4me3 occupancy after stress treatment (Sani et al., 2013). The authors primed *Arabidopsis* seedlings by exposing the roots for 24 h to osmotic stress (NaCl), and 10 days later applied a second stress in the form of drought or salt stress to the plants. During this subsequent 10-day growth period, the primed plants were indistinguishable from the untreated control; however, they showed a significantly higher tolerance to the second stress, indicating a somatic memory of the priming event (Sani et al., 2013). Immediately after priming, the distribution of H3K27me3 and H3K4me3 occupancy was analyzed in seedlings. H3K27me3 changed toward shorter and more fragmented ‘islands’, and H3K27me3 genome coverage was reduced (Sani et al., 2013). In addition, H3K4me3 occupancy was increased in some regions, indicating a more ‘active’ chromatin structure immediately after priming. The observed preferential loss of H3K27me3 at some TF encoding genes was assumed to enable a rapid activation of stress-responsive genes upon a second stress. However, the expected correlation between mRNA levels and changes in

chromatin marks was not apparent directly after priming. Genes carrying increased levels of H3K27me3 did not show a significant decrease in transcription, and genes carrying elevated H3K4me3 did not show a significant increase in corresponding mRNA levels, indicating that changes in these chromatin marks do not correlate with transcriptional changes, at least under the conditions tested. The observed rapid reduction of H3K27me3 occupancy after 24 h of stress exposure suggests an active demethylation of H3K27me3, as only limited cell division will occur during this time frame (Sani et al., 2013). Interestingly, expression of the rice H3K27me2/3 demethylase *JMJ705* is induced by stress signals and during pathogen infection (Li et al., 2013). Furthermore, JMJ705 preferentially removes H3K27me3 from genes responding to biotic stress, thereby revealing an essential role of a histone demethylase in stress and defense responses (Li et al., 2013). To analyze immediate changes of H3K27me3 and H3K4me3 occupancy after priming, Sani et al. (2013) used roots, organs that were challenged with NaCl. The analysis of shoots, which are not directly exposed to the stress and may only be primed by systemic signaling, did not show significant alterations in H3K4me3 (H3K27me3 was not analyzed) directly after priming (Sani et al., 2013). To analyze the somatic memory of the priming stimulus, the genome-wide H3K27me3 profile (but not the H3K4me3 profile) was determined 10 days after priming in whole roots. Interestingly, the changes in H3K27me3 levels were sustained at 102 genes at this stage (Sani et al., 2013). Nevertheless, this pattern may not necessarily rely on epigenetic mechanisms [according to the definition by Russo et al. (1996)]: first, in the 10-day growth period after priming, a large proportion of cells will not divide, so the analyzed tissue presents a mixture of 'old' and newly formed cells. It can therefore not be distinguished whether changes in histone modifications are preserved in cells that have been exposed to the stress or whether they are also 'inherited' in newly formed root cells. Second, after a 2-week drought stress treatment, the primed plants' shoots showed higher drought resistance than shoots of non-primed plants. It would now be interesting to analyze whether the enhanced resistance is due to signaling from root to shoot (and would therefore not be epigenetic) or due to epigenetic marks established in shoot cells after priming. Vernooij et al. (1994) revealed that SAR can be established in scions that were grafted onto primed rootstocks after the priming event, thus demonstrating that the perception of a stress is communicated from root to shoots even after the initial stress ceased (Vernooij et al., 1994).

The existence of signaling from primed to non-primed cells that induces chromatin modification in the non-primed cells is strongly supported by work from Jaskiewicz et al. (2011). Seventy-two hours after priming of *Arabidopsis* lower leaves by a chemical or the bacterium *Pseudomonas syringae*, non-stressed upper leaves showed increased levels of H3K4me3 at the promoter of the TF genes *WRKY29*, *WRKY6*, and *WRKY53* with only little induction of their transcription, hence poising/priming these genes for rapid induction upon a subsequent stress (Jaskiewicz et al., 2011) (see Figure 2). After a second stress, the upper leaves of the primed plants showed a much stronger increase in transcription of these genes compared with the non-primed plants (Jaskiewicz et al., 2011). Thus, systemic signaling can induce a somatic memory of a stress during SAR. It will be highly interesting to identify the nature of the (systemic) signal leading to durable stress resistance and chromatin modifications upon stress. This signal may regulate PcG and TrxG protein activity or targeting and/or activate 'erasers' of epigenetic modifications.

Transgenerational memory of stress

To address transgenerational memory of stress, several studies analyzed whether priming can affect the resistance of subsequent generations to abiotic and biotic stress. H3K27me3 occupancy and its potential role in transgenerational memory of SAR were analyzed by Luna et al. (2012). The authors showed that the progeny (P1) of pathogen-infected plants (P0) exhibited an enhanced H3K27me3 level at the promoter of *PLANT DEFENSIN1.2* (*PDF1.2*), a gene encoding a host defense peptide (Luna et al., 2012). The *npr1-1* (*non-expressor of PR1*) mutant, which is deficient in SAR, failed to enrich H3K27me3 at *PDF1.2* in the P1 generation and comprised a reduced defense response, indicating that NPR1 is important for transgenerational SAR (Luna et al., 2012). Increased resistance in the wild type P1 may not necessarily rely on epigenetic inheritance but may be the result of signal transmission from the mother plant to the embryo. Therefore, the authors also analyzed the P2, which was either derived from stressed or non-stressed P1 plants. Importantly, stress treatment in the P0 and a stress-free P1 was sufficient to increase pathogen resistance in P2, indicating epigenetic, transgenerational inheritance (Luna et al., 2012). However, another study could not confirm this result, but revealed that only the offspring (P1) of primed plants showed higher resistance to pathogens (Slaughter et al., 2012). Similarly, another study observed increased tolerance to stress only in the offspring (P1) of primed plants, but not over a stress-free generation (Boyko et al., 2010). Thus, priming conditions and type of stimulus

may strongly influence heritability of stress memory over several generations. Similarly, by testing the effect of 10 different chemical and physical stress treatments on the frequency of somatic homologous recombination (SHR), a DNA repair pathway regulated by epigenetic mechanisms (reviewed in Schuermann et al., 2005), it was revealed that most of the stress treatments led to increased SHR in the stressed plants, but only to low and stochastic increase in SHR in the two subsequent non-stressed generations (Pecinka et al., 2009). Another study analyzed the effect of radiation (UV-C) on SHR and showed that the SHR frequency was significantly increased in all four subsequent non-stressed generations (Molinier et al., 2006). Thus, also here, experimental setup likely determines whether stress exposure may lead to transgenerational memory and potentially may have adaptive consequences. It will therefore be important to determine the underlying differences in experimental setup. In addition, we suggest that observation of transgenerational memory of stress should be repeated in different laboratories.

Conclusion

Epigenetic gene regulation has an important impact to suppress the expression of costly defense genes in the absence of stress, but likely also enables plants to remember a stress in somatic tissues and the following generations.

A given stress is usually not perceived by all cells of a plant; however, a signal generated by stress may be propagated or spread into present but non-challenged or newly emerging organs involving long-range signaling and/or epigenetic mechanisms. This course of events is already known for SAR, a process in which an organ is challenged and the information is spread to organs that did not directly encounter the stress stimulus. This spreading of information was shown to depend on plant hormones, volatile compounds, and non-proteinaceous amino acids. Systemic spreading can result in changes in chromatin marks that are thought to poise genes for later induction, when a second stress is experienced. It is not yet known how the stress memory is induced; however, it likely involves writers and erasers of chromatin modifications. In the future, it will therefore be important to analyze SAR and chromatin changes in PcG, TrxG, and histone demethylase mutants to reveal whether these proteins are involved in acquired resistance.

Loss of PcG or TrxG function can lead to either increased or decreased stress tolerance, depending on the analyzed mutant. This is not surprising as PcG and TrxG proteins target thousands of genes that likely include genes involved in conferring or suppressing stress resistance. Owing to their broad spectrum of target genes, PcG and TrxG mutants display pleiotropic phenotypes, which can make the interpretation of their function in stress responses difficult. Hence, it would be useful to analyze stress-induced knockdown mutants of PcG and TrxG proteins to avoid secondary effects due to the pleiotropy of the respective mutants. Currently, changes in histone modifications and stress resistance phenotypes of plants lacking the respective writers have not been well connected. Therefore, the analysis on how PcG and TrxG act on stress tolerance remains incomplete, and evidence for a role of PcG and TrxG proteins in somatic and transgenerational stress memory is currently lacking.

Analysis of stress-induced changes of the chromatin modifications H3K27me3 and H3K4me3, mediated by PcG and TrxG proteins, respectively, was extensively performed. The results are difficult to interpret because the distinction between stress-induced chromatin marks and stable epigenetic marks is highly dependent on the experimental setup. Epigenetic marks, which are sustained during mitosis and/or meiosis and are important for somatic and transgenerational memory, need to be monitored over a longer time span and in more organs than was currently performed. Thus, at the moment, there is no conclusive evidence that stress-induced chromatin marks are stably inherited during cell divisions and can therefore be called epigenetic.

Acknowledgments: J.A.K. and D.S. are supported by a 'PLUS3' grant from the Boehringer Ingelheim Foundation. We apologize to colleagues whose work was not cited due to space limitations.

Funding: Boehringer Ingelheim, (Grant/Award No. 'PLUS3').

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1.3.2 ER-stress and the unfolded protein response (UPR) in plants

During development cells experience different states of gene expression, sometimes extensive gene expression can cause an accumulation of unfolded or misfolded proteins in the Endoplasmic reticulum (ER), leading to ER-stress. Additionally, biotic and abiotic stress such as pathogen infection, high salt, and heat, can cause ER-stress (Che et al., 2010; Deng et al., 2011; Gao et al., 2008; Liu et al., 2007b; Liu et al., 2011a; Moreno et al., 2012). Unfolded ER proteins are degraded by ER-associated protein degradation (ERAD), a process involving re-localization of proteins into the cytoplasm where they are degraded in a 26S-proteasome-dependent manner (reviewed in Deng et al., 2013a). Specialized proteins in the ER sense the unfolded proteins and activate the unfolded protein response (UPR) if too many unfolded proteins accumulate. The UPR in *Arabidopsis* has two "arms", one involving IRE1 (Inositol Requiring Enzyme 1), an ER transmembrane ribonuclease kinase which senses unfolded proteins in the ER lumen (Gardner and Walter, 2011), and the other involving the ER transmembrane TF bZIP28 (Liu et al., 2007a) (Figure 1. 3). The simultaneous loss of IRE1 and bZIP28 was shown to be lethal, emphasizing the importance of functional UPR during development (Deng et al., 2013b). During ER-stress, IRE1 unconventionally splices *bZIP60* mRNA in the cytoplasm (Deng et al., 2011; Nagashima et al., 2011). Splicing of *bZIP60* mRNA results in soluble bZIP60 protein which can enter the nucleus to activate downstream UPR genes (Deng et al., 2011; Iwata et al., 2008; Nagashima et al., 2011; Zhang et al., 2015). Under ER-stress conditions bZIP28 relocates from the ER to the Golgi where it is proteolytically processed; its cytoplasmic bZIP-containing domain subsequently enters the nucleus (Liu et al., 2007a; Srivastava et al., 2013). bZIP60 and bZIP28 can heterodimerize (Liu and Howell, 2010)

and are mostly functionally redundant in UPR (Sun et al., 2013a), but they also bind target genes independently (Liu and Howell, 2010; Sun et al., 2013b).

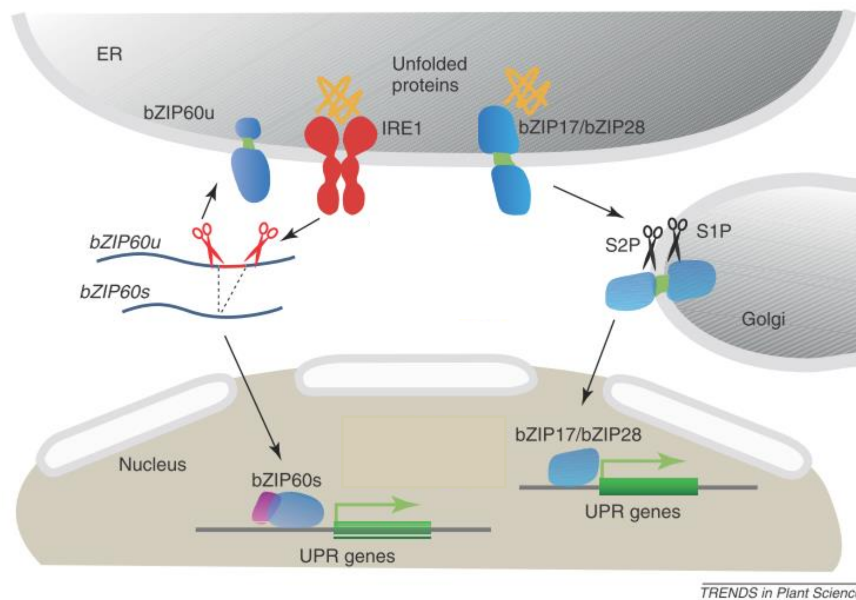

Figure 1. 3: Model of the plant unfolded protein response (UPR).
IRE1 and bZIP28 sense unfolded proteins in the ER lumen. When bound by unfolded proteins, IRE1 unconventionally splices *bZIP60* mRNA, enabling processed bZIP60 to enter the nucleus. Upon ER-stress, bZIP28 is transported from the ER to the Golgi where it is proteolytically processed. Its N-terminal bZIP-containing domain subsequently enters the nucleus. Both bZIP60 and bZIP28 activate downstream UPR genes in the nucleus, together and independently. Figure modified from Iwata and Koizumi (2012).

Early during ER-stress, UPR inhibits transcription and translation, activates genes which help the cell to deal with an excess of unfolded or misfolded proteins, such as the chaperone BIP3, and induces genes which prevent programmed cell death (PCD) such as *BI1* (*BAX INHIBITOR 1*) (Watanabe and Lam, 2008). When ER-stress is prolonged or exceeds the protein folding capacity of the ER, PCD will be induced (Watanabe and Lam, 2008). Until now no negative regulator of ER-stress responses has been identified in plants.

ER-stress responses can induce changes in histone modifications. In animals and humans, the H3K18Ac deacetylase SIRT7 was shown to repress transcription of ribosomal proteins in response to ER-stress, in order to prevent accumulation of unfolded proteins until ER homeostasis is reestablished (Shin et al., 2013). H4R3 methylation and H4 acetylation were shown to be induced by ER-stress at the *GRP78/BIP* promoter in human cell lines, thereby activating *GRP78/BIP* expression (Baumeister et al., 2005). The H3K4me3-binding protein SGF29 plays a central and dual role in the ER-stress response. Prior to ER-stress, the protein coordinates H3K4me3 levels, thereby maintaining a 'poised' chromatin state on ER-stress target gene promoters (Schram et al., 2013). Following ER-stress induction, SGF29 is required for increased H3K14 acetylation on these genes, which then results in full transcriptional

activation, thereby promoting cell survival (Schram et al., 2013). The role of chromatin modifications in ER-stress responses in plants is only emerging. A study by Song et al. (2015) revealed that ER-stress induced the deposition of H3K4me3 at the PcG target genes *SEC31A* and *BIP3*. This deposition was shown to be mediated by the COMPASS-like complex, which is targeted to these genes by bZIP60 and bZIP28, and which recruits H3K4 methyltransferases to these loci (Song et al., 2015). In the same study it was also shown that the ER-stress-responsive genes *NSF*, *ERDJ3A*, *SARA1A* and *TIN1* did not acquire H3K4me3 during ER-stress, revealing that not all ER-stress-responsive genes are targeted by H3K4me3 for induction. Therefore, the activation of ER-stress-responsive gene expression appears to be regulated by binding of TFs alone, or in concert with H3K4 methyltransferases.

1.4 Aims of this study

1.4.1 Elucidating the role of BLI in PRC2-mediated gene silencing

The role of BLI in PRC2-mediated gene silencing is not well understood. Previous analysis of several ectopically expressed H3K27me3 target genes in *bli-1* mutants revealed that H3K27me3 levels at those genes were unchanged. It was previously shown that PRC2-mediated H3K27 trimethylation is not sufficient for gene silencing (Schubert et al., 2006). Additional proteins are required for stable repression of certain H3K27me3 target genes and BLI is likely one of them. To understand whether BLI regulates the expression of a specific class of H3K27me3 target genes, and whether it has PRC2 related and unrelated functions, the transcriptional profile of *bli-1* mutants will be analyzed in this study. Chromatin immunoprecipitation (ChIP) will reveal whether the histone methylation status of certain, highly mis-regulated H3K27me3 target genes -identified by transcriptional profiling- is changed in *bli-1*. This analysis will help to understand whether the loss of *BLI* affects H3K27me3 only at a subset of genes in *Arabidopsis*. The action of PRC2 can be counteracted by TrxG proteins, and loss of PRC2 leads to increased H3K4me3 levels (Lafos et al., 2011). To reveal if loss of *BLI* potentially interferes with TrxG function, H3K4me3 levels at certain H3K27me3 target genes will be analyzed by ChIP. Moreover, genome-wide H3K4me3 levels in *bli* mutants will be analyzed by immunoblots.
Additionally, the expression and localization of CLF will be analyzed in *bli* mutants to reveal if BLI might regulate CLF on the genic or the protein level.

1.4.2 The role of BLI in stress response regulation

BLI was shown to be an important regulator of cold-responsive gene expression (Purdy et al., 2010). The transcriptional profiling of *bli-1* mutants will reveal if *BLI* is involved in the

regulation of additional stress responses. To understand how BLI regulates stress responses, *bli* mutants will be exposed to several stress conditions. As mentioned above, stress can induce changes in chromatin modifications. Therefore, the chromatin status of several stress-regulated genes will be analyzed by ChIP to understand if loss of *BLI* alters histone methylation marks (H3K27me3 and H3K4me3) at stress-responsive genes. Therefore, BLI might link the PcG system to stress responses in *Arabidopsis*.

1.4.3 Analysis of BLI subcellular localization

BLI-GFP was shown to localize to nuclei and cytoplasmic speckles (Purdy et al., 2010; Schatlowski et al., 2010). To determine the identity of these speckles, several fluorescent marker proteins for cytoplasmic compartments such as the Golgi, the ER, and processing-bodies, will be tested for colocalization with BLI-GFP.

To reveal which domains are responsible for the localization of BLI-GFP in nuclei and cytoplasmic speckles, it will be analyzed how BLI mutations affect its subcellular localization in *Arabidopsis*. For this purpose BLIs nuclear import signal (NLS) and nuclear export signal (NES) will be mutated. Additionally, a viral (SV40) NLS will be added to analyze how constitutive nuclear localization of BLI affects plant growth. The ability of mutated BLI to complement the strong *bli-1* phenotype will be tested as well.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in this study were supplied by the following companies:
AppliChem (Darmstadt), Biozym (Hessisch Oldendorf), Duchefa (Haarlem, NL), Eurogentech (Köln), Invitrogen (Karlsruhe), Merck (Darmstadt), Roche (Mannheim), Roth (Karlsruhe), Serva (Heidelberg), Sigma-Aldrich (Steinheim), Thermo Fisher Scientific (Schwerte), VWR (Darmstadt).

2.1.2 Buffer and Media

Buffer and Media were prepared according to protocols by Ausubel (1996) and Sambrook et al. (1989), if not stated otherwise.

Bacteria:	LB	1 % NaCl, 0.5% yeast extract, 1 % bacto tryptone, pH 7.0; 1 % agar for solid media
	YEB	0.5 % sucrose, 0.1 % yeast extract, 0.5 % bacto peptone, 0.5 % beef extract, 2 mM $MgCl_2$, pH 7.2; 1 % agar for solid media
Plants:	½ MS	2.2 g MS (per 1L), 0.05 % MES; 0.5 % sucrose; pH 5.7 0.8 % plant agar for solid media

2.1.3 Enzymes

All used restriction enzymes for analytical or preparative cleavage of DNA were ordered from Thermo Fisher Scientific or New England Biolabs (NEB) (Frankfurt a. M.).
All other enzymes and enzyme mixes were ordered from Thermo Fisher Scientific, Invitrogen (Karlsruhe), NEB, and Eurogentech, and used according to the manufacturer´s instructions.

2.1.4 Antibodies

Table 2.1: Antibodies used for Chromatin immunoprecipitation (ChIP) and Immunoblot experiments.
*: The CLF (S110) antibody was kindly provided by Justin Goodrich and detects the N-terminal part of GFP-CLF fusion proteins. ab: antibody

Name	Purpose	Source	Dilution	Supplier
anti-H3K27me3	ChIP	rabbit	1:1000	Diagenode
anti-H3K4me3	ChIP/ Immunoblot	rabbit	1:1000/1:5000	Diagenode
anti-igG	ChIP	rabbit	1:1000	Diagenode
anti-CLF (S110)	Immunoblot (primary ab)	sheep	1:2000	J. Goodrich*
anti-sheep (HRP)	Immunoblot (secondary ab)	rabbit	1:10,000	Santa Cruz
anti-H3	Immunoblot (primary ab)	mouse	1:2000	Diagenode
anti-mouse (IRDye 680RD)	Immunoblot (secondary ab)	rabbit	1:15,000	Li-Cor
anti-rabbit (IRDye 800CW)	Immunoblot (secondary ab)	rabbit	1:7000	Li-Cor

2.1.6 Oligonucleotides

All oligonucleotides used in this study are listed in the tables below. Oligonucleotides designed for this study were ordered from Eurogentech (Liege, BE).

Table 2.2: Oligonucleotides used for genotyping mutant plant lines.

Purpose	Sequence forward (5´-3´)	Sequence reverse (5´-3´)
bli-1	TATCCCACGGTTCTTTTTGG	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
bli-11	TATCCCACGGTTCTTTTTGG	ATATTGACCATCATACTCATTGC
BLI	GCACTGGCAGAATCCTTAGC	TATCCCACGGTTCTTTTTGG
GFP	GTGCTGCAAGGCGATTAAGT	TGTAACAGCGCAGAAGATGG
***BLI* (plants with gBLI transgene)**	GCACTGGCAGAATCCTTAGC	GGAACTCTTCAAGTTCATGGTGTTTCTCAC

Table 2.3: Oligonucleotides used for Chromatin immunoprecipitation (ChIP) using H3K4me3 and H3K27me3 antibodies. *: designed by Kwon et al. (2009); **: designed by Song et al. (2015).

gene name	ATG number	Sequence forward (5´-3´)	Sequence reverse (5´-3´)
H3K27me3 ChIP			
PI	AT5G20240	CCACATATCCTCTCCTCCATA	CCATTCCTCCTCTTTGAGAACG
SEP2	AT2G21970	TGTTTTTGATGCGTGAGGTT	CAAAGCTCTGTTGGCATCAA
SEP3	AT1G24260	GGGTTTCCAATTTTGGGTTT	GATGAATCCCATCCCCAAGT
AG	AT4G18960	TGGGTACTGAGAGGAAAGTGAGA	GGATCGTAGAAGGCAGACCA
BIP3	AT1G09080	GTGAGCTTGCGAAACGATCT	CCTCGAATCTTGCTCTCGTT
SEC31A	AT1G18830	TACAAGGAAGCAGTGGCTCA	CCCACAATTCTGTACCACCA
LTP2	AT2G38530	GCAACGGCGTTACTAACCTT	TTTAGCGGCAGATTGAAGGC
-	AT1G17960	CTTCCGGCTTGCTTCAAACT	AGATCCCAACACCGCACTAT
-	AT3G55700	TTCAACCCCATGATCGAGCT	AGAAGGATCGGGGAAGTTGT
ACT7	AT5G09810	TAGTGAAAAATGGCCGATGG	CCATTCCAGTTCCATTGTCA
FUS3*	AT3G26790	GTGGCAAGTGTTGATCATGG	AGTTGGCACGTGGGAAATAG
H3K4me3 ChIP			
SEC31A-P**	AT1G18830	GACAACACACAAATGACGTG	GAGAGTGACTCGAAGAAAGC
SEC31A-B**	AT1G18830	GAACTCGATTTTCAGTCCAA	TTGGATTCCATAAACCGATG
NSF**	AT4G21730	GTCTAGCCAATCAGAGAATG	ACGTACACAAATGTTATGGC
NAC103	AT5G64060	AACTTGGCACCTGGTTTTCG	AATGTCGACCTCAGCAATGG
BIP3-P**	AT1G09080	TGTCACGTGTCTGCTTGTGA	TAGCCTCGGTAGAGTGTCCT
BIP3-B**	AT1G09080	CACGGTTCCAGCGTATTTCAAT	ATAAGCTATGGCAGCACCCGTT
ERDJ3A**	AT3G08970	GTGAGTAATTGCCCCTACCA	CTTCCTCTTCTAAGCGTGTC
SARA1A**	AT1G09180	TAAACTCTCCTGGGTCCTGG	ACACGTGGGTAATGGGGACT
TIN1**	AT5G64510	GGCGAAGCCATTGTCAATAC	GGTTTTCACGGGAAGAGATG
ACT7	AT5G09810	TAGTGAAAAATGGCCGATGG	CCATTCCAGTTCCATTGTCA

Table 2.4: Oligonucleotides used for gene expression analysis by quantitative RT-PCR.
*: reference gene described by Czechowski et al. (2005).

gene name	ATG number	Sequence forward (5´-3´)	Sequence reverse (5´-3´)
bZIP60	AT1G42990	GATGATGACGAAGAAGGAGACG	TCTAACCGCCGCATCTCTAT
bZIP28	AT3G10800	TCCGCATTCAACAGCTCTCT	AACTGGAAAACCTCGGTGCA
BIP3	AT1G09080	GGTGAAGGTGGAGAAGAAACAC	CCTCCGACAGTTTCAATACCGA
NAC103	AT5G64060	CCATTGCTGAGGTCGACATT	ACCACTTAAGATCTCCAGTCCC
NAC089	AT5G22290	AGGCGAAAGAACTGACTGGA	AACCCGGCAAACAACCATAG
BLI	AT3G23980	AGAGGGAACATTTCCCTCTG	GAAACTGCTCAAGCTTACGG
ACT7	AT5G09810	CCAGGAATTGCTGACCGTAT	GGTGCAACCACCTTGATCTT
CLF	AT2G23380	TTTCGATAACCTGTTCTGCC	GTCTCCCACTACCTTTCACC
PP2A-1*	AT1G59830	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA

Table 2.5: Oligonucleotides used for site directed mutagenesis of *cBLI*.
Sequences of mutated nucleotides and NLS tag (PKKKRKV) are underlined. Mutated amino acids and respective replacements are indicated.

primer name	Sequence (mutated nucleotides and NLS tag are underlined)	mutation
BLI-cDNA_mutNLS_MUT	CTTGAGCAGTTTCGTGCTCGAGCTGCAGCAGAAAAAGCT	K(25)A, K(27)A
BLI-cDNA_mutNLS_R	CTTACGGCGTCCAGCTTCAACGTC	-
BLI-cDNA_mutCyc_MUT	GTTGAAGCTGGACGCTGGAAGAAAGAGCAGTTTCGTAAA	R(18)W, L(20)K
BLI-cDNA_mutCyc_R	GTCCTCTTGCCTCCGGGAACTAGT	-
BLI-cDNA_mutNES_MUT	GATTTTTCTAATAGCAAGGCCCGAATAGGTTCATCGAAG	L(116)K, E(117)A, L(118)R
BLI-cDNA_mutNES_R	AACTTTGCCCACTGATTCCTGACC	-
BLI-cDNA_mutSPEK_MIM	GCCATAGACAATGTTGATCCAGAAAAGCAGCAG	S(665)D
BLI-cDNA_mutSPEK_DePh	GCCATAGACAATGTTGTACCAGAAAAGCAGCAG	S(665)V
BLI-cDNA_mutSPEK_R	CATCTTCTGCGCTGTTACAAGCTC	-
NLS-tag cBLI_F	ATGGGGCCCAAGAAAAAGCGCAAGGTTATGGCATCAG CTACTAGTTCCCGG	PKKKRKV
NLS-tag cBLI_R	AGCCTGCTTTTTGTACAAACTTGG	-

2.1.7 Molecular size standards

For the determination of DNA sizes, using agarose gel-electrophoresis, the following DNA ladders were used: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific), GeneRuler™ 100 bp DNA Ladder (Thermo Fisher Scientific), and 1 kb DNA Ladder (NEB). For protein gel electrophoresis and immunoblots PageRuler Plus Pre-stained Protein Ladder (Thermo Fisher Scientific) and Chameleon Duo Pre-stained Protein Ladder (Li-Cor) were used.

2.1.8 Vectors

Table 2.6: List of vectors used in this study.

Vector name	construct	backbone
empty vectors		
pGKGWG (Zhong et al., 2008)	GFP	
pABindGFP (Bleckmann et al., 2010)	i35S:C-termGFP	
entry vectors		
pDONR201_cBLI-STOP (Schatlowski et al., 2010)	cBLI w/o Stop codon	pDONR201
pDONR201_cBLI-mutNLS	cBLI_mutNLS	pDONR201
pDONR201_cBLI-mutCYC	cBLI_mutCyc	pDONR201
pDONR201_cBLI-mutNES	cBLI_mutNES	pDONR201
pDONR201_cBLI-mutSPEK.phosphomimic	cBLI_SPEK.MIM	pDONR201
pDONR201_cBLI-mutSPEK.dephospho	cBLI_SPEK.DePh	pDONR201
pDONR201-NLS-cBLI	NLS-cBLI	pDONR201
pDONR201-NLS-cBLI_mutNLS	NLS-cBLI_mutNLS	pDONR201
destination vectors		
pGKGWG-proBLI	BLI promoter	pGKGWG
pGKGWG-proBLI-cBLI	BLI:cBLI-GFP	pGKGWG
pGKGWG-proBLI-cBLI-mutNLS	BLI:cBLI_mutNLS-GFP	pGKGWG
pGKGWG-proBLI-cBLI-mutNES	BLI:cBLI_mutNES-GFP	pGKGWG
pGKGWG-proBLI-cBLI-SPEK.MIM	BLI:cBLI_SPEK.MIM-GFP	pGKGWG
pGKGWG-proBLI-cBLI-SPEK.DePh	BLI:cBLI_SPEK.DePh-GFP	pGKGWG
pGKGWG-proBLI-NLS-cBLI	BLI:NLS-cBLI-GFP	pGKGWG
pGKGWG-proBLI-NLS-cBLI_mutNLS	BLI:NLS-cBLI_mutNLS-GFP	pGKGWG
pAB117-cBLI	i35S:cBLI-GFP	pABindGFP
pAB117-cBLI-mutNLS	i35S:cBLI_mutNLS-GFP	pABindGFP
pAB117-cBLI-mutCyc	i35S:cBLI_mutCyc-GFP	pABindGFP
pAB117-cBLI-mutNES	i35S:cBLI_mutNES-GFP	pABindGFP
pAB117-cBLI-SPEK.MIM	i35S:cBLI_SPEK.MIM-GFP	pABindGFP
pAB117-cBLI-SPEK.DePh	i35S:cBLI_SPEK.DePh-GFP	pABindGFP

2.1.8 Bacteria strains

Table 2.7: Bacteria strains used for amplifications of plasmid DNA and for plant transformation.

bacteria	purpose	genotype
E. coli		
DH5α	plasmid amplification and cloning	F^- *end*A1 *thi*-1 *rec*A1 *rel*A1 *sup*E44 *pho*A *gyr*A96 Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsd*R17(r_K^- m_K^+), λ–
DB3.1	amplification of vectors containing the ccdB gene	F-, *gyr*A462, *end*A1, (sr1-recA), *mcr*B, *mrr, hsd*S20, Δ(r B -, m B -), *sup*E44, *ara*-14, *gal*K2, *lac*Y1, *pro*A2, *rps*L20, (Sm R), *xyl*-5, λ-*leu*, *mtl*1
A. tumefaciens		
GV3101 (pMP90)	transformation of *A. thaliana*	C58C1, pMK90, Rif^r, $Gent^r$ (Koncz and Schell, 1986)
GV3101 (p19)	transformation of *N. benthamiana*	C58C1, Rif^r (Koncz and Schell, 1986) Contains p19 silencing suppressor

2.1.9 Plant material

Seeds of the *Arabidopsis thaliana* ecotype Columbia (Col-0) were ordered form the European Arabidopsis Stock Centre (NASC, N1092).

2.1.9.1 *A. thaliana* T-DNA insertion lines

Table 2.8: *A. thaliana* T-DNA insertion lines used in this study.
If needed, SAIL, SALK and GABI-Kat T-DNA insertion lines were selected on PPT, Kan and Sulf, respectively.

gene	accession no	allele	T-DNA insertion	NASC no.
BLI	AT3G23980	*bli-1*	SAIL_107_D04	N805222
BLI	AT3G23980	*bli-2*	SALK_005565	N505565
BLI	AT3G23980	*bli-3*	SAIL_518_E07	N821933
BLI	AT3G23980	*bli-11*	GABI-Kat_663H12	-
CLF	AT2G23380	*clf-28*	SALK_139371	N639371

2.1.9.2 Transgenic *A. thaliana* lines

Table 2.9: Transgenic *A. thaliana* lines used in this study.
Wt: wild type. i35S: ß-estradiol inducible promoter. *: obtained by cross with *clf-50/35S:GFP-CLF* (Schubert et al., 2006).

Name	Vector/construct	Back-ground	Ecotype
bli-1/BLI:BLI-GFP	pGKGWG-gBLI	*bli-1*	Col-0
bli-11/BLI:BLI-GFP	pGKGWG-gBLI	*bli-11*	Col-0
bli-1/BLI:cBLI-GFP	pGKGWG-proBLI-cBLI	*bli-1*	Col-0
bli-1/BLI:cBLI_mutNLS-GFP	pGKGWG-proBLI-cBLI-mutNLS	*bli-1*	Col-0
bli-1/BLI:cBLI_mutNES-GFP	pGKGWG-proBLI-cBLI-mutNES	*bli-1*	Col-0
bli-1/BLI:cBLI_SPEK.MIM-GFP	pGKGWG-proBLI-cBLI-SPEK.MIM	*bli-1*	Col-0
bli-1/BLI:cBLI_SPEK.DePh-GFP	pGKGWG-proBLI-cBLI-SPEK.DePh	*bli-1*	Col-0
bli-1/BLI:NLS-cBLI-GFP	pGKGWG-proBLI-NLS-cBLI	*bli-1*	Col-0
bli-1/BLI:NLS-cBLI_mutNLS-GFP	pGKGWG-proBLI-NLS-cBLI_mutNLS	*bli-1*	Col-0
bli-11/BLI:cBLI-GFP	pGKGWG-proBLI-cBLI	*bli-11*	Col-0
bli-11/BLI:cBLI_mutNLS-GFP	pGKGWG-proBLI-cBLI-mutNLS	*bli-11*	Col-0
bli-11/BLI:cBLI_mutNES-GFP	pGKGWG-proBLI-cBLI-mutNES	*bli-11*	Col-0
bli-11/BLI:cBLI_SPEK.MIM-GFP	pGKGWG-proBLI-cBLI-SPEK.MIM	*bli-11*	Col-0
bli-11/BLI:cBLI_SPEK.DePh-GFP	pGKGWG-proBLI-cBLI-SPEK.DePh	*bli-11*	Col-0
bli-11/BLI:NLS-cBLI-GFP	pGKGWG-proBLI-NLS-cBLI	*bli-11*	Col-0
bli-11/BLI:NLS-cBLI_mutNLS-GFP	pGKGWG-proBLI-NLS-cBLI_mutNLS	*bli-11*	Col-0
i35S::cBLI-GFP	pAB117-i35S-BLI-GFP	Wt	Col-0
i35S::N/SMC-BLI-GFP	pAB117-i35S-N/SMC-BLI-GFP	Wt	Col-0
i35S::SMC-GFP	pAB117-i35S-SMC-GFP	Wt	Col-0
i35S::GFP-CLF-ΔSET	pMDC7-i35S- GFP-CLF-ΔSET (M. L. Hohenstatt)	Wt	Col-0
BIP3:GUS (Maruyama et al., 2010)	BIP3:GUS	Wt	Col-0
H2B-RFP (De Rybel et al., 2010)	35S:H2B-RFP	Wt	Col-0
WAK2-RFP (Nelson et al., 2007)	35S:WAK2-RFP	Wt	Col-0
SYP32-RFP/WAVE22 (Geldner et al., 2009)	UBQ10:SYP32-RFP	Wt	Col-0

VTI12-RFP/WAVE13 (Geldner et al., 2009)	UBQ10:VTI12-RFP	Wt	Col-0
*bli-1/35S:GFP-CLF**	*35S:GFP-CLF*	*bli-1*	Col-0/WS
*bli-11/35S:GFP-CLF**	*35S:GFP-CLF*	*bli-11*	Col-0/WS
clf-50/35S:GFP-CLF (Schubert et al., 2006)	*35S:GFP-CLF*	*clf-50*	WS

2.1.10 Software and online resources

2.1.10.1 Software

AxioVision Rel. V4.8, Zeiss

LSM Image Browser V4.2, Zeiss

Zen blue edition, Zeiss

Vector NTI 10.3.0, Invitrogen

2.1.10.2 Online resources

Analysis of Arabidopsis mutant lines

TAIR	http://arabidopsis.org/
T-DNA express	http://signal.salk.edu/cgi-bin/tdnaexpress

Microarray analysis

VirtualPlant 1.3	http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/
GOToolbox	http://genome.crg.es/GOToolBox/

Protein domain analysis

ExPASy bioinf. resource portal	http://prosite.expasy.org/
PredictProtein	http://www.predictprotein.org
NLS prediction	http://www.moseslab.csb.utoronto.ca/NLStradamus/
NES prediction	http://www.cbs.dtu.dk/services/NetNES/

Protein modification analysis

PhosPhAt 4.0	http://phosphat.uni-hohenheim.de/
SMART	http://smart.embl-heidelberg.de/
PlantsP	http://plantsp.genomics.purdue.edu/myrist.html

Protein Subcellular Localization Prediction

PSORT	http://psort.hgc.jp/form.html

Sequence analysis and Cloning

Arabidopsis methylation browser	http://epigenomics.mcdb.ucla.edu/H3K27m3/
BLAST	http://www.arabidopsis.org/Blast/
	http://blast.ncbi.nlm.nih.gov/Blast.cgi
ClustalW2	http://www.ebi.ac.uk/Tools/msa/clustalw2/
Primer 3	http://bioinfo.ut.ee/primer3-0.4.0/primer3/

2.2 Methods

2.2.1 Plant growth conditions

Arabidopsis thaliana

Seeds were sterilized for 10 min in 70% Ethanol supplemented with 0.05% Triton X-100, and for 10 min in 96% Ethanol. Sterile seeds were sown on 1/2 MS germination medium: half-strength Murashige and Skoog medium including vitamins (Duchefa), supplemented with 0.5% sucrose, 0.05% MES (2-(n-morpholino)-ethanesulfonic acid), and 0.8% plant agar for solid medium. For selection of transgenic lines, antibiotics were added to solid medium. Seeds were stratified for two days at 4°C and grown under long day conditions, (8/16 h dark/light rhythm at 20 °C). *bli-1* and *bli-11* seeds showed a germination delay of two days (Schatlowski et al., 2010). Therefore, when directly compared, these two genotypes were sown two days earlier than all other genotypes, stratified for two days at 4°C, and then transferred to the respective growth condition. After 10-14 days seedlings were transferred to soil, if indicated.

Nicotiana benthamiana

Plants were grown on soil under long day conditions.

2.2.2 Plant transformation:

Arabidopsis thaliana

bli-1 and *bli-11* heterozygous mutants were transformed using the floral-dip method (Clough and Bent, 1998) and *Agrobacterium tumefaciens* strain GV3101 pMP90 (Koncz and Schell, 1986).

Nicotiana benthamiana

N. benthamiana leaves were transformed as described in Bleckmann et al. (2010) using *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) containing the silencing suppressor p19.

2.2.2.1 Induction of transient transgene expression in plants

Induction of expression in *Arabidopsis* was obtained by inoculation of seedlings with 10 µM ß-estradiol for 12 hours. *N. benthamiana* leaves were brushed with 20 µM beta-estradiol + 0.1% Tween 20, 24 h prior to imaging.

2.2.3 Stress experiments

2.2.3.1 Drought stress treatment

For detailed description please see Materials and Methods section of Manuscript I.

2.2.3.2 ER-stress treatment

For detailed description please see Materials and Methods section of Manuscript II.

2.2.4 Chlorophyll measurement

For detailed description please see Materials and Methods section of Manuscript II.

2.2.5 Chromatin immunoprecipitation (ChIP)

For detailed description please see Materials and Methods section of Manuscript I.

2.2.6 Microarray analysis and Principal Component Analysis (PCA)

For detailed description please see Materials and Methods section of Manuscript I.

2.2.7 GUS staining

For detailed description please see Materials and Methods section of Manuscript II.

2.2.8 Basic molecular methods

2.2.8.1 Cloning and vector generation

PCR amplification of templates was performed using Phusion Polymerase (Thermo Fisher Scientific). Mutagenesis of vectors was achieved using the Phusion site directed mutageneis Kit (Thermo Fisher Scientific) according to the manufacturer´s instructions. All cloning reactions were performed by either cleaving DNA by restriction enzymes (NEB and Thermo Fisher Scientific) followed by T4-Ligase (Thermo Fisher Scientific) mediated re-ligation, or by using GATEWAY technology (Invitrogen). Oligonucleotides and vectors used for cloning are displayed in Table 2.5 and Table 2.6. Details on Cloning of destination vectors can be found in the Material and Methods section of Manuscripts I and II in this study.

2.2.8.2 Isolation, quantification, and analysis of plasmid DNA

Plasmid DNA was isolated from *E. coli* cells using QIAprep Spin Miniprep Kit (Qiagen, Hilden) and peqGold X Change Plasmid Midi Kit (Peqlab, Erlangen). DNA concentration was determined using the Qubit system by Invitrogen. Verification of plasmids was achieved by restriction analysis (enzymes from NEB or Thermo Fisher Scientific). DNA was sequenced by GATC to verify cloning success and to reveal possible mutations.

2.2.8.3 Isolation of genomic DNA from *A. thaliana*

Genomic DNA was isolated following a modified protocol according to Dellaporta et al. (1983).

2.2.8.4 Isolation of RNA from *A. thaliana*

RNA was extracted using innuSPEED Plant RNA Kit (Analytik Jena), resuspended in 30 µl RNAse-free water, and treated with DNaseI (Thermo Fisher Scientific). RNA concentration was determined using NanoDrop technology (Thermo Fisher Scientific).

2.2.8.5 Synthesis of cDNA

cDNA was synthesized from 1 µg RNA by use of RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific) and Oligo(dT) oligonucleotides. The obtained cDNA was diluted 1:10, and 2 µl of this dilution were used for RT-qPCR.

2.2.8.6 Quantitative PCR

qPCR analysis was performed with technical triplicates and at least two biological replicates using oligonucleotides listed in

Table 2.3 and Table 2.4. KAPA SYBR FAST qPCR Master Mix was used according to the manufacturer's instructions in a 2-step PCR program (95°C 5:00 min, 40 x (95°C 0:15 min, 60°C 0:30 min), melting curve: 65-95 °C in 0.5 °C steps) in one of the following qPCR machines: Light Cycler 480 (Roche), CFX384 Touch™ Real-Time PCR Detection System (Biorad), or Stratagene Mx3005P (Agilent Technologies), using the respective software for evaluation. Expression levels were normalized to the reference gene PP2A-1 (AT1G59830) (Czechowski et al., 2005), if not stated otherwise. Normalization of ChIP experiments is described in the Material and Methods section of Manuscript I.

2.2.8.7 Protein isolation from *A. thaliana*

100 mg plant material were ground to a fine powder in liquid nitrogen (lN_2). Samples were taken out of lN_2 and thawn for 10-15 sec. Then 200 µl of 95°C hot 2xLaemmli buffer (150 mM Tris pH 6.8, 3.3 % SDS, 30% glycerol, 15% ß-mercaptoethanol, 0.0018% bromophenol blue) were added, samples were vortexed for 10 sec, and put into lN_2. Samples were heated at 95°C for 10 min and then centrifuged at 10,000 rpm for 10 min at RT. The supernatant was transferred to a new reaction tube and centrifugation was repeated. Protein concentration was quantified using Amidoblack (see 2.2.8.7.2). Samples were stored at -70°C.

2.2.8.7.2 Histone isolation from *A. thaliana*

0.5-2 g plant material were ground to a fine powder in liquid nitrogen (lN_2). The powder was re-suspended in 30 ml extraction buffer 1 (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 5 mM ß-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM proteinase inhibitor cocktail (Sigma-Aldrich)) and filtered through two layers of Miracloth (VWR). The filtered solution was centrifuged for 20

min, at 2100xg at 4°C. The supernatant was removed and the pellet was re-suspended in 1 ml of extraction buffer 2 (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 5 mM ß-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM proteinase inhibitor cocktail (Sigma-Aldrich), 1% Triton X-100). The solution was transferred to a 1.5 ml reaction tube, incubated for 5 min on ice, and centrifuged for 10 min at 12,000xg, at 4°C. The nuclei-pellet was re-suspended in 1 ml extraction buffer 2, incubated for 5 min on ice, and centrifuged for 10 min at 12,000xg, at 4°C. The nuclei were re-suspended in 400 µl 0.4 N H_2SO_4, and incubated rotating at 4°C, O/N. Then, samples were centrifuged for 10 min at 16,000xg, at 4°C, and the supernatant was transferred to a fresh reaction tube. 100% Trichloroacetic acid were added to the sample to a final concentration of 33%. Samples were incubated for 30 min on ice and then centrifuged for 10 min at 16,000xg, at 4°C. The supernatant was removed, the pellet was washed three times with 1 ml of cold acetone, and then centrifuged for 10 min at 16,000xg, at 4°C. The pellet was dried for 5 min at RT, re-suspended in 100 µl 1xLaemmli buffer, and heated for 10 min at 95°C. Protein concentration was quantified using Amidoblack (see 2.2.8.7.2). Samples were stored at -70°C.

2.2.8.7.2 Protein concentration determination using Amidoblack

500 µl of Amidoblack solution (90% Methanol, 10% acetic acid, 0.01% (w/v) Amidoblack (Roth)) were added to 10 µl protein sample. Samples were incubated for 3 min at RT and then centrifuged at 14,000 rpm for 15 min, at RT. The supernatant was removed and the pellet was washed with 1 ml wash buffer (90% Methanol, 10% acetic acid). The reaction tube was inverted 10 times and then centrifuged for 10 min at 14,000 rpm at RT. The wash buffer was removed and the pellet was air-dried for 5-7 min. The pellet was re-suspended in 500 µl 0.2 N NaOH. Absorption was measured at 600 nm and concentration was calculated according to a BSA (bovine serum albumin) calibration curve.

2.2.8.8 Immunoblot procedures

The immunoblot procedures were performed as described in Ausubel (1996). Protein extracts were incubated at 95 °C for 5 min before loading on 10% (15% for histones) SDS Polyacrylamide gels. Gel Electrophoresis was performed using a Mini-PROTEAN tetra cell (BioRad). Proteins were transferred to a PVDF membrane (Roth) using a Trans-Blot SD semi dry transfer cell (BioRad). Primary antibodies were incubated O/N at 4°C, and secondary antibodies for 2 h, at RT. SuperSignal West Femto Maximum Sensitivity Substrat (Thermo Fischer Scientific) was used for detection of chemiluminescence produced by the HRP coupled secondary antibody in an ImageQuant LAS4000 mini (GE Healthcare Life Sciences). After imaging, the membrane was stained for 1h with 0.1% Ponceau S solution (Sigma-Aldrich) in

5% acetic acid, then wash 5 min in 5% acetic acid. For fluorescence detection, membranes were imaged using the Li-Cor Odyssey Classic imaging system (Li-Cor Biotechnology).

2.2.9 Microscopy

Differential interference contrast (DIC) microscopy

Plants in this study were analysed and imaged using a Zeiss Stereomicroscope (Stemi 2000-C) equipped with AxioCam ICc1 (Zeiss) or using a Nikon Stereomicroscope SMZ25.

Confocal Laser Scanning microscopy

Confocal LSM was performed using LSM 780 and LSM 510 microscopes (Zeiss). A 40x1.20 C-Apochromat water-immersion objective was used for imaging with LSM 780 and for LSM 510 a 40×1.3 numerical aperture oil-immersion objective was used. Image acquisition was carried out sequentially to prevent crosstalk between channels. Images were processed using ZEN software and LSM Image Browser (Zeiss), respectively.

Table 2.10: Excitation and Emission spectra of fluorophores used in this thesis.
BP: bandpass; LP: longpass

Fluorophore	Excitation	Emission LSM 510	Emission LSM 780
GFP	488 nm, argon laser	Meta channel 505-550 nm	510-550 nm
RFP	561 nm, diode	Meta channel 571-636 nm	575-630 nm
DAPI	405 nm, diode	BP 420-480 nm	420-480 nm
PI	561 nm, diode	LP 575 nm	575-620 nm

3 Results

3.1 Manuscript I

As an interactor of the PRC2 methyltransferase CLF, BLI is likely involved in the epigenetic gene regulation by H3K27 trimethylation. However, previous analysis of several ectopically expressed H3K27me3 target genes in *bli-1* mutants revealed that H3K27me3 levels at those genes were unchanged, which is in contrast to PRC2 mutants. It was shown before that PRC2-mediated H3K27 trimethylation is not sufficient for gene silencing (Schubert et al., 2006). Additional proteins are required for stable repression of certain H3K27me3 target genes and BLI is likely one of them. To understand whether BLI regulates the expression of a specific class of H3K27me3 target genes, and whether it has PRC2 related and unrelated functions, the transcriptional profile of *bli-1* mutants was analyzed in this study. A significant number of H3K27me3 target genes was mis-regulated in *bli-1* mutants, revealing its importance in PRC2-mediated gene silencing. Analysis of the histone methylation status of highly up-regulated H3K27me3 target genes revealed that loss of *BLI* did not affect H3K27me3 levels, but H3K4me3 levels at these genes. This indicated a role for BLI in the control of gene expression downstream of, or in parallel to, PRC2.

Additionally, the transcriptional profile of *bli-1* mutants revealed that loss of *BLI* function led to a strong mis-regulation of genes regulated in response to drought, heat, high salt, cold, endoplasmic reticulum (ER-) stress, the phytohormone abscisic acid (ABA), and systemic acquired resistance (SAR). To understand how *bli* mutants respond to stress treatment, they were exposed to drought stress conditions. The stress treatment revealed that *bli* mutants are hypersensitive to drought stress and indicates that BLI is required for stress resistance and stress responses regulation.

These results are described in detail in Manuscript I "BLISTER regulates Polycomb-target genes and is involved in the negative regulation of specific stress responses in *Arabidopsis thaliana*" in this work.

"BLISTER regulates Polycomb-target genes and is involved in the negative regulation of specific stress responses in *Arabidopsis thaliana*"

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Keywords: Polycomb (PRC2), abiotic stress, H3K27me3, drought, ABA, CLF, BLI

Author contributions:

J.A. Kleinmanns, N. Schatlowski and D. Schubert designed the research. N. Schatlowski prepared RNA samples and performed the microarray experiment. D. Schubert performed SEM microscopy. D. Heckmann processed the microarray data and performed the PCA. J.A. Kleinmanns evaluated the microarray, i.e. compared gene lists, generated diagrams and performed GO term analysis, performed ChIP and stress experiments, and analysed GUS reporter lines. J.A. Kleinmanns wrote the manuscript, which was revised by D. Schubert.

Abstract

Epigenetic gene regulation by Polycomb group (PcG) proteins is mediated by post-translational modification of histones. The POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) silences its target genes by trimethylating H3K27 (H3K27me3). Previously we identified the plant-specific protein BLISTER (BLI) as an interactor of the PRC2 methyltransferase CURLY LEAF (CLF). We showed that BLI regulates several PcG target genes but is also likely to have PcG-independent functions, such as preventing premature differentiation and promotion of cell division. An independent study revealed that *BLI* is needed for cold tolerance. To further understand the function of *BLI,* we analyzed the transcriptional profile of *bli-1* mutants in a microarray experiment. Approximately 40% of the up-regulated genes in *bli-1* are PcG target genes, and a significant number of these genes is regulated by the phytohormone abscisic acid (ABA). Importantly, *bli-1* mutants did not show changes in H3K27me3 levels at all tested genes, indicating that BLI regulates PcG target genes downstream of PRC2. Furthermore, genes involved in meristem identity (*CLV3*) and cell cycle regulation (*CYCB1;1*) are ectopically active in *bli-1*, which is consistent with its proposed function in maintaining cell identity. Genes involved in responses to abiotic stress such as drought, high salinity, or heat stress, and genes up-regulated by systemic acquired resistance (SAR) were overrepresented among the up-regulated genes in *bli-1*. Consistently, the loss of *BLI* reduced drought stress tolerance, indicating that *BLI* is involved in the positive regulation of drought stress responses. We conclude that *BLI* is a key regulator of stress-responsive genes: it represses ABA-responsive PcG target genes, likely downstream of PRC2, and promotes drought and cold stress resistance of *Arabidopsis*.

Introduction

Epigenetic gene regulation is mediated by several mechanisms such as histone modifications or DNA methylation. Polycomb group proteins assemble in large complexes and maintain gene repression. The POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) consists of four core members and silences target genes by trimethylation of histone 3 lysine 27 (H3K27me3). In *Arabidopsis thaliana*, PRC2 is composed of one of three SET domain-containing histone methyltransferases MEDEA (MEA), SWINGER (SWN), and CURLY LEAF (CLF); one of three VEFS domain-containing proteins EMBRYONIC FLOWER 2 (EMF2), FERTILIZATION INDEPENDENT SEED 2 (FIS2), and VERNALIZATION 2 (VRN2); and the two WD40 domain-containing proteins FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) (reviewed in

Derkacheva and Hennig, 2014). The loss of PRC2 function leads to a loss of H3K27me3 at PcG target genes which may be associated with ectopic expression of those genes. Trithorax group (TrxG) proteins, such as ARABIDOPSIS TRITHORAX1 (ATX1) (Alvarez-Venegas et al., 2003), ULTRAPETALA1 (ULT1) (Carles and Fletcher, 2009), and BRAHMA (BRM) (Farrona et al., 2004), act antagonistically to PRC2. TrxG proteins activate gene expression through setting H3K4me3 and by ATP-dependent chromatin remodeling. The other PcG complex is PRC1, which is composed of the subunits LHP1 (LIKE-HETEROCHROMATIN PROTEIN1), EMF1 (EMBRYONIC FLOWER 1), AtRING1A (At RING FINGER PROTEIN1A), AtRING1B, AtBMI1A (B-cell specific Moloney murine leukaemia virus insertion site1), AtBMI1B, and AtBMI1C, and silences genes by H2A monoubiquitination and chromatin remodeling (Beh et al., 2012; Bratzel et al., 2010; Bratzel et al., 2012; Calonje et al., 2008; Chen et al., 2010; Turck et al., 2007; Xu and Shen, 2008; Yang et al., 2013). The exact composition of the plant PRC1 is not yet clear but, like in mammals and *Drosophila*, presence of several PRC1 complexes, which repress genes by H2Aub-dependent and -independent mechanisms, is indicated (reviewed in Merini and Calonje, 2015). It was long assumed that PcG recruitment follows a hierarchical order. In this model PRC2 sets H3K27me3, which is recognized and bound by PRC1, PRC1 in turn monoubiquitinates H2A leading to chromatin compaction and stable gene silencing. This model was first proven to be wrong in plants (Yang et al., 2013) and later on in vertebrates (Blackledge et al., 2014; Cooper et al., 2014; Kalb et al., 2014). In the current model PRC1 takes the lead: PRC1 binds to target genes, monoubiquitinates H2A and recruits PRC2 to set H3K27me3 (reviewed in Merini and Calonje, 2015). Importantly, PRC2 is also targeted independently of PRC1, e.g. by interaction with transcription factors and likely by non-coding RNAs (Heo and Sung, 2011; Liu et al., 2011b; Lodha et al., 2013), therefore the current hierarchical recruitment model is rather true for certain- but not all- PcG target genes.

As plants are sessile organisms, they need to rapidly respond to stress, e.g. by altered gene expression and metabolite production. Plant stress responses that result in osmotic imbalance and cell desiccation, such as drought, high salinity, and cold, involve the phytohormone abscisic acid (ABA). Early in development ABA regulates seed maturation and maintains seed dormancy. During vegetative development ABA is involved in general growth and reproduction and plays an important role in the response to stress (reviewed in Tuteja, 2007). Although ABA plays an important role in the drought, high salinity, and cold stress responses, these stresses are also regulated by ABA-independent pathways. Previously it was shown that the PRC2 component MSI1 is a negative regulator of drought stress response (Alexandre et al.,

2009). Recently a study revealed that MSI1 functions in a histone deacetylase complex to fine-tune ABA signaling and that loss of MSI1 led to an increased tolerance to salt stress (Mehdi et al., 2015). The levels of H3K27me3 were not analyzed in both studies (Alexandre et al., 2009; Mehdi et al., 2015), therefore it remains unclear if the PcG function of MSI1 plays a role in the regulation of stress-responsive genes. Interestingly, loss of CLF results in a reduced resistance to drought (Liu et al., 2014) suggesting that different PRC2 members have distinct functions in regulating stress responses or that the role of MSI1 in drought stress regulation is due to its function in additional complexes.

We previously showed that CLF is interacting with the plant-specific protein BLISTER (BLI) (Schatlowski et al., 2010). *BLI* is ubiquitously expressed throughout development and its loss results in a strong pleiotropic phenotype with mutants displaying affected seed, leaf, and flower development and a strong reduction in plant size. We previously showed that BLI regulates expression of several PcG target genes but likely also has PcG-independent functions (Schatlowski et al., 2010).

Here, using transcriptional profiling of *bli-1* mutants, we revealed that a significant number of PcG target genes is mis-regulated and that a significant number of those genes is regulated by ABA. Importantly, de-repression of PcG target genes in *bli-1* is not due to reduced H3K27me3 levels, indicating a role of BLI downstream of PRC2 function. Furthermore, we report that in *bli-1* mutants a high number of stress-responsive genes is mis-regulated and that *bli-1* mutants display a reduced tolerance to drought stress. We propose that BLI is not only involved in the positive regulation of drought stress but might function as a general regulator of stress responses which is achieved in part by regulating stress-responsive PcG target genes.

Results

To further understand whether BLI predominantly regulates PcG target genes, we performed a microarray experiment using a 44k Agilent array. We used *bli-1* seedlings grown for 12 days under continuous light conditions and compared the transcriptional profile to the Col-0 wild type.

Transcriptional profiling reveals a functional overlap of BLI and CLF target genes

In our microarray experiment we could detect 292 up- and 244 down-regulated genes in *bli-1* seedlings (Figure 1) (TOP 25 up-regulated genes in Table 3; full list in Supplemental data 1). As BLI interacts with CLF and *bli-1 clf-28* double mutants revealed a synergistic genetic interaction (Schatlowski et al., 2010), we analyzed the overlap of mis-regulated genes in *bli-1*

and *clf-28* mutants (Farrona et al., 2011). We found a significant overlap of mis-regulated genes between the two mutants (Figure 1) (Supplemental data 2). Importantly, *CLF* is not mis-regulated in *bli-1* (Supplemental Figure 2) and *BLI* is not mis-regulated in *clf-28* (Farrona et al., 2011). Among the commonly up-regulated genes in *bli-1* and *clf-28* are several Pc-G target genes (10 out of 18), e.g. the MADS-box transcription factor gene *SEPALLATA3* (*SEP3*). However, a large number of genes was only mis-regulated in one of either mutant. Because *CLF* function is masked by partial redundancy with *SWN*, we also compared the overlap of genes mis-regulated in *bli-1* and the strong *clf-28 swn-7* (*clf swn*) double mutant (Farrona et al., 2011), which is completely deficient in PRC2 function (Chanvivattana et al., 2004; Lafos et al., 2011). The overlap of genes up- regulated in *bli-1* and *clf swn* was significant (Figure 1 C); among the 62 overlapping genes 37 are targeted by PRC2. The overlap of down-regulated genes in *bli-1* and *clf swn* was also significant (Figure 1 D); almost half of the down-regulated genes in *bli-1* were also down-regulated in *clf swn*, revealing a strong co-regulation of genes by BLI, CLF, and SWN. Among the 101 commonly down-regulated genes in *bli-1* and *clf swn*, 53 were PRC2 target genes. Our data hence reveal that a subset of genes targeted by CLF and/or SWN are co-regulated by BLI. Importantly, BLI also regulates genes in a PcG-independent manner.

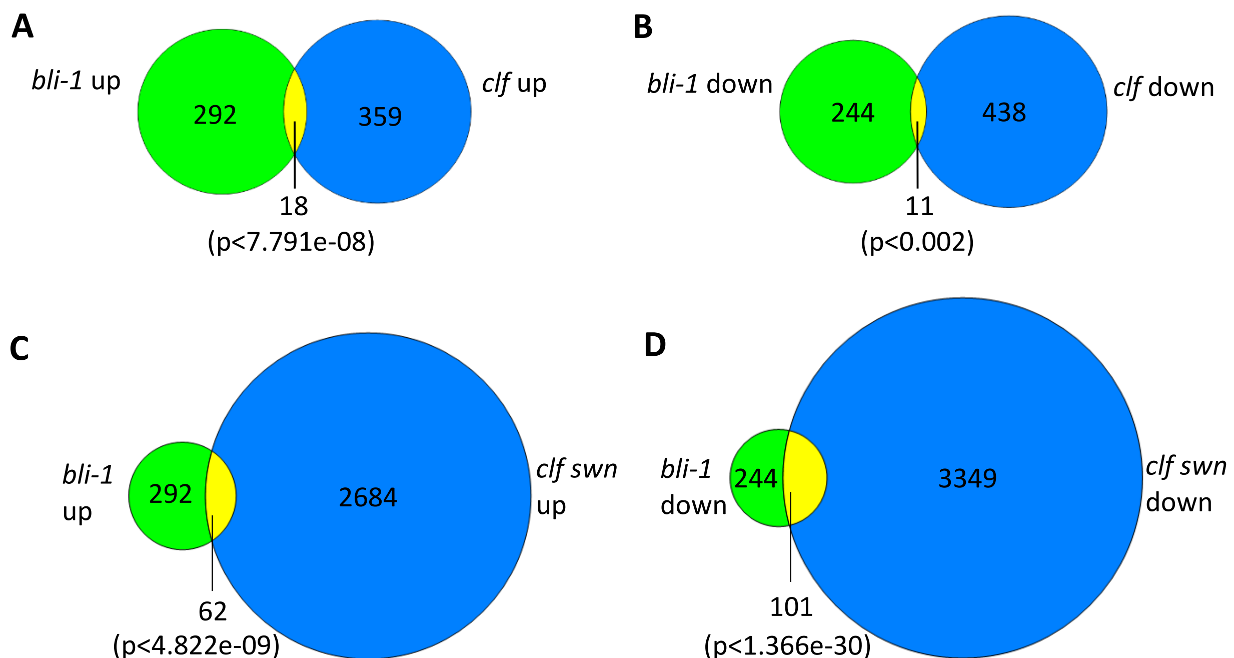

Figure 1: Venn diagrams of mis-regulated genes in *bli-1* compared to *clf-28* and *clf swn* double mutants.
A) Comparison of up-regulated genes in *bli-1* seedlings vs. up-regulated genes in *clf-28* (Farrona et al., 2011). B) Comparison of down-regulated genes in *bli-1* seedlings vs. down-regulated genes in *clf-28*. The comparison of mis-regulated genes in *bli-1* and *clf-28* revealed a significant overlap between the two mutants. C) Comparison of *bli-1* and *clf swn* up-regulated genes. D) Comparison of *bli-1* and *clf swn* down-regulated genes. The overlap of *bli-1* and *clf swn* mis-regulated genes was highly significant. Statistical significance was tested using the hypergeometric distribution; a p-value below 0.05 was considered as statistically significant.

bli-1 mutants show a mis-regulation of PcG target genes but no loss of H3K27me3

To further understand the role of BLI in PcG-mediated gene regulation, we compared the *bli-1* mis-regulated genes to PcG (H3K27me3) target genes. Indeed, we identified a significant number of PcG target genes mis-regulated in *bli-1* seedlings (Table 1, Supplemental data 3), but no mis-regulation of PRC2 members (Supplemental data 1). To further address the role of *BLI* in PcG mediated gene repression and reveal possible changes in H3K27me3 levels at mis-regulated genes, we performed Chromatin immunoprecipitation (ChIP) (Figure 2 A). For ChIP experiments we used *bli-1*, *clf-28,* and the complemented lines *bli-1/BLI:BLI-GFP* and *bli-11/BLI:BLI-GFP* (data not shown). Furthermore, we used the novel *bli-11* mutant, which strongly resembles *bli-1,* as an internal control to exclude possible T-DNA-dependent effects on *bli-1* chromatin modifications (for characterization of *bli-11* see Supplemental Figure 3). We determined H3K27me3 levels at MADS-box transcription factor genes *PI* (*PISTILLATA*), *SEP2* (*SEPALLATA2*), and *SEP3*, which are well known Pc-G target genes and are up-regulated in *bli-1*. Moreover, we determined H3K27me3 levels at several highly up-regulated Pc-G target genes in *bli-1*: *BIP3* (*BINDING PROTEIN3*), *SEC31A* (*SECRETORY31A*), At3g55700, At1g17960, and *LTP2* (*LIPID TRANSFER PROTEIN2*) (Table 3). *AG* (*AGAMOUS*) is one of the main target gene of CLF and carries reduced H3K27me3 levels in *clf* mutants leading to ectopic expression (Goodrich et al., 1997; Schubert et al., 2006). In our ChIP experiments, both *bli* mutants did not show significant changes in H3K27me3 levels at most analyzed loci; only *bli-11* showed moderately decreased H3K27me3 levels at *SEP3*. *clf-28* showed significantly reduced H3K27me3 levels at *AG*, as expected, but not at other loci. In summary, we could not detect reduced levels of H3K27me3 at the tested loci in *bli-1* and *bli-11* mutants, despite a strong de-repression of these genes in *bli-1*, suggesting that mis-regulation of these genes is independent or downstream of H3K27me3. However, it is possible that changes in chromatin modifications at the tested loci are only occurring in specific tissues, which we would not detect in our analysis using whole seedlings.

Table 1: H3K27me3 target genes mis-regulated in *bli-1* seedlings.
*: Genome wide H3K27me3 target genes refer to data from (Oh et al., 2008). **: total number of protein coding genes according to TAIR8 genome release. Statistical significance was tested by Chi square test with Yates correction; a p-value equal to or below 0.05 was considered as statistically significant.

	PcG (H3K27me3) targets	total no. genes	percentage of H3K27me3 targets	Chi square test (p-value)
genome wide (Oh et al., 2008)	7832*	27235**	28.76	
***bli-1* up+down**	208	536	38.81	0.0003
***bli-1* up**	109	292	37.33	0.0241
***bli-1* down**	98	244	40.16	0.0064

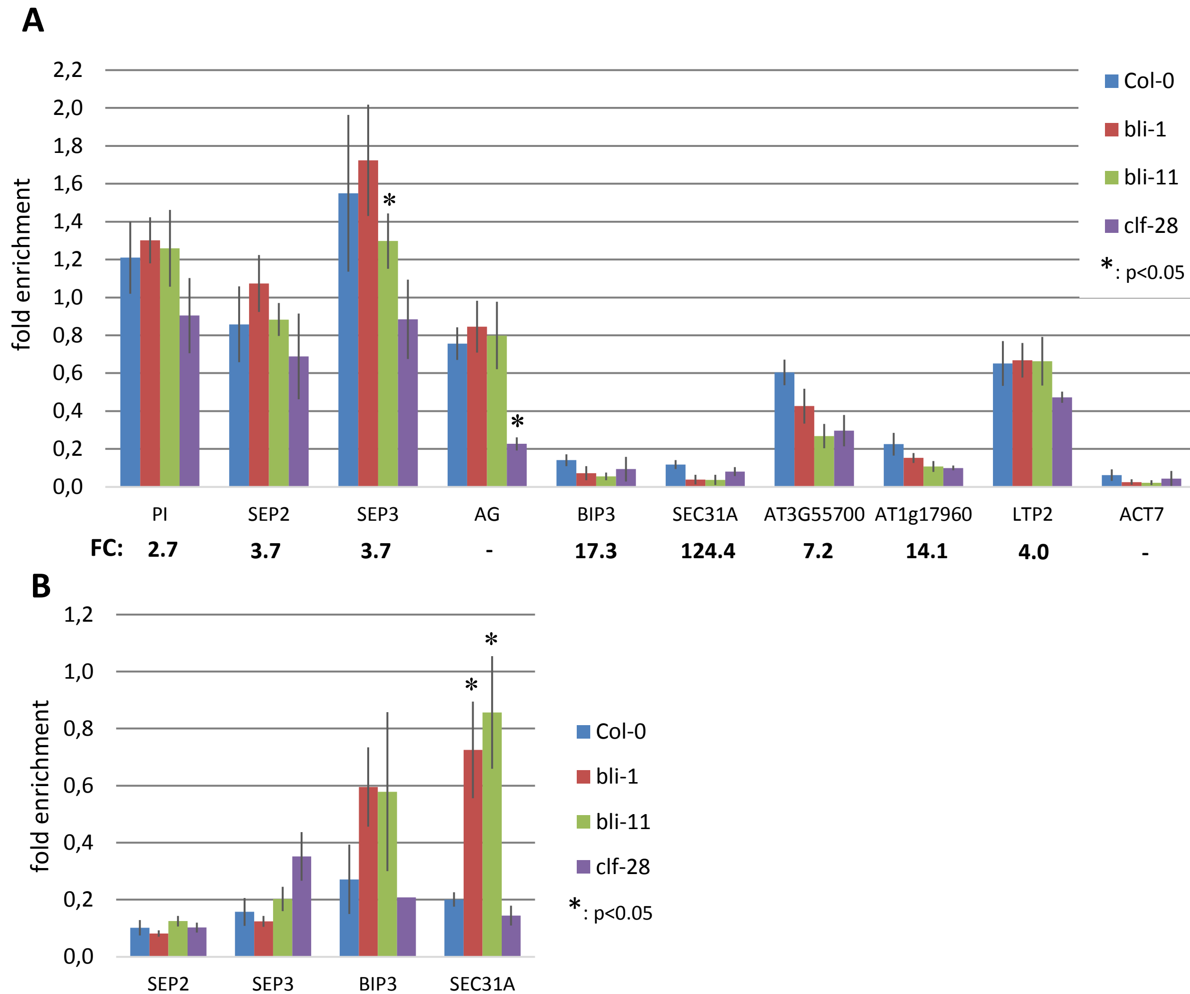

Figure 2: Chromatin immunoprecipitation (ChIP) in *bli* and *clf* mutants.
A) H3K27me3 levels at Polycomb target genes in 14 day old seedlings. Chromatin was precipitated using H3K27me3 antibodies and was amplified by quantitative PCR using oligonucleotides binding inside the gene body. H3K27me3 levels at each locus were normalized to the *FUS3* locus. FC: fold-change in expression level in *bli-1* compared to the wild type. B) H3K4me3 levels at Polycomb target genes in 14 day old seedlings. Chromatin was precipitated using H3K4me3 antibodies and was amplified by quantitative PCR using oligonucleotides binding near the transcriptional start site. H3K4me3 levels at each locus were normalized to the *ACT7* locus. All ChIP experiments were performed twice with 2 biological and 3 technical replicates, respectively, and showed similar results. Error bars indicate ±SE of 2 independent experiments. Test for statistical significance by Student´s t-test; a p-value below 0.05 was considered as statistically significant.

The action of PcG proteins is counteracted by Trithorax group (TrxG) proteins, which set the activating H3K4me3 mark. H3K4me3 targets a much higher number of genes (approximately 1/2 of the Arabidopsis genome) than H3K27me3 (Bouyer et al., 2011; Oh et al., 2008; Roudier et al., 2011; Zhang et al., 2009), but also shares many target genes with H3K27me3, e.g. *AG* (Saleh et al., 2007). To test if the increased expression of PcG-target genes in *bli-1* mutants is due to increased activity of TrxG proteins - hence elevated levels of H3K4me3 at those genes - we performed a ChIP experiment using antibodies directed against H3K4me3 (Figure 2 B). For H3K4me3 ChIP experiments we analyzed two PcG target genes carrying a high level of H3K27me3 (*SEP2*, *SEP3*) and two carrying a low H3K27me3 level (*BIP3*, *SEC31A*). Only the highest expressed gene in *bli-1*, *SEC31A* (Table 3), showed increased H3K4me3 levels (Figure

2 B), suggesting that BLI prevents H3K4me3 accumulation on at least a subset of PcG target genes. Generally, genes targeted by H3K4me3 were not enriched among mis-regulated in *bli-1* (Table 2).

Table 2: H3K4me3 target genes mis-regulated in *bli-1* seedlings.
*: Genome wide H3K4me3 target genes refer to data from Roudier et al. (2011). **: total number of protein coding genes according to TAIR8 genome release. Statistical significance was analyzed by Chi square test with Yates correction; a p-value equal to or below 0.05 was considered as statistically significant.

	H3K4me3 targets	total no. genes	Percentage of H3K4me3 targets	Chi-square test (p-value)
genome wide (Roudier et al., 2011)	17836	27235*	65.49	
***bli-1* up+down**	313	536	58.40	0.1182
***bli-1* up**	172	292	58.90	0.2938
***bli-1* down**	141	244	57.79	0.2600

Table 3: Top 25 up-regulated genes in *bli-1* 12 day old seedlings.
Yes/no in the far-right column indicates if gene is an H3K27me3 target or not; Asterisks indicate genes that were tested for H3K27me3 (*) and H3K4me3 (**) coverage in ChIP experiments.

#	Symbol	Description	Fold change	H3K27me3 target
1	AT1G18830	Transducin/WD40 repeat-like superfamily protein; Secretory 31A (SEC31A)	124.35	yes**
2	AT4G21730	pseudogene of N-ethylmaleimide sensitive factor (NSF)	48.15	no
3	AT5G64060	NAC domain containing protein 103 (NAC103)	23.48	no
4	AT5G55270	Protein of unknown function (DUF295)	18.52	yes
5	AT1G09080	Heat shock protein 70 (Hsp 70) family protein, Binding protein 3 (BIP3)	17.31	yes**
6	AT1G17960	Threonyl-tRNA synthetase	14.13	yes*
7	AT3G08970	DNAJ heat shock N-terminal domain-containing protein, (ERDJ3A)	12.39	no
8	AT2G29350	senescence-associated gene 13 (SAG13)	10.90	yes
9	AT5G53230	Protein of unknown function (DUF295)	10.73	yes
10	AT5G53240	Protein of unknown function (DUF295)	9.96	yes
11	AT1G09180	secretion-associated RAS super family 1 (SARA1)	8.44	no
12	AT3G57260	beta-1,3-glucanase 2, PATHOGENESIS-RELATED PROTEIN 2, (PR2)	8.00	yes
13	AT2G38240	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	7.83	yes
14	AT3G17050	transposable element gene	7.65	yes
15	AT3G55700	UDP-Glycosyltransferase superfamily protein	7.17	yes*
16	AT5G64510	Tunicamycin-induced 1 (TIN1)	7.04	no
17	AT1G21528	unknown protein	6.87	no
18	AT1G27020	unknown protein	6.64	yes
19	AT3G28899	unknown protein	6.43	no
20	AT5G41761	unknown protein	6.26	yes
21	AT5G26270	unknown protein	6.14	no
22	AT1G42990	basic region/leucine zipper motif 60 (bZIP60)	5.77	no
23	AT3G53232	ROTUNDIFOLIA like 1 (RTF1)	5.73	yes
24	AT1G56060	unknown protein	5.62	no
25	AT1G72280	endoplasmic reticulum oxidoreductins 1 (ERO1)	5.37	no

bli* plants show additional expression domains of *CLV3* and *CYCB1;1

In the severe *clf swn* double mutant H3K27me3 is completely lost (Lafos et al., 2011). Cell fate decisions in this mutant cannot be maintained throughout development, leading to a loss of cell identity and the formation of callus-like tissue (Chanvivattana et al., 2004). Presence of blister-like structures in *bli-1* (Schatlowski et al., 2010) and *bli-11* mutants (Supplemental Figure 3 F and G) indicate a loss of cell identity in *bli* mutants. The blister-like structures may have meristematic activity or are actively dividing cells in an otherwise differentiated tissue. Strikingly, transcriptional profiling of *bli-1* did not reveal changes in the expression of the stem cell marker *CLV3* (*CLAVATA 3*) and the cell division marker *CYCB1;1* (*CYCLIN-DEPENDENT PROTEIN KINASE B1;1*). Mis-regulation of a gene in a small population of cells might not be detected when whole seedlings are used for transcriptional profiling. To test if this could be the case for *bli-1*, we analyzed the expression pattern of a *CLV3:GUS* and a *CYCB1;1:GUS* reporter. The *bli-1* mutant cannot be used for the analysis of *GUS* expression patterns, because it shows an ectopic expression of the *LAT52:GUS* marker gene present on the SAIL T-DNA (Schatlowski et al., 2010). Therefore, we used *bli-11* to study *GUS* expression patterns of *CLV3* and *CYCB1,1* (Figure 3). *CLV3:GUS* showed ectopic expression in 43% (32 out of 74 seedlings) of *bli-11* seedlings, mainly in hypocotyls and cotyledons. *CYCB1;1:GUS* was also ectopically expressed in *bli-11* (32%, 8 of 25 seedlings), particularly in differentiated leaves in which *CYCB1;1* expression has seized in wild type plants. Expression of both reporters was confined to a limited number of cells, which may reflect blister-like structures or de-differentiating cells. Overall, ectopic expression of the stem cell marker *CLV3* and the cell division marker *CYCB1;1* in *bli-11* mutants indicate that *BLI* acts in maintaining cell identity and in suppression of improper or ectopic cell-divisions.

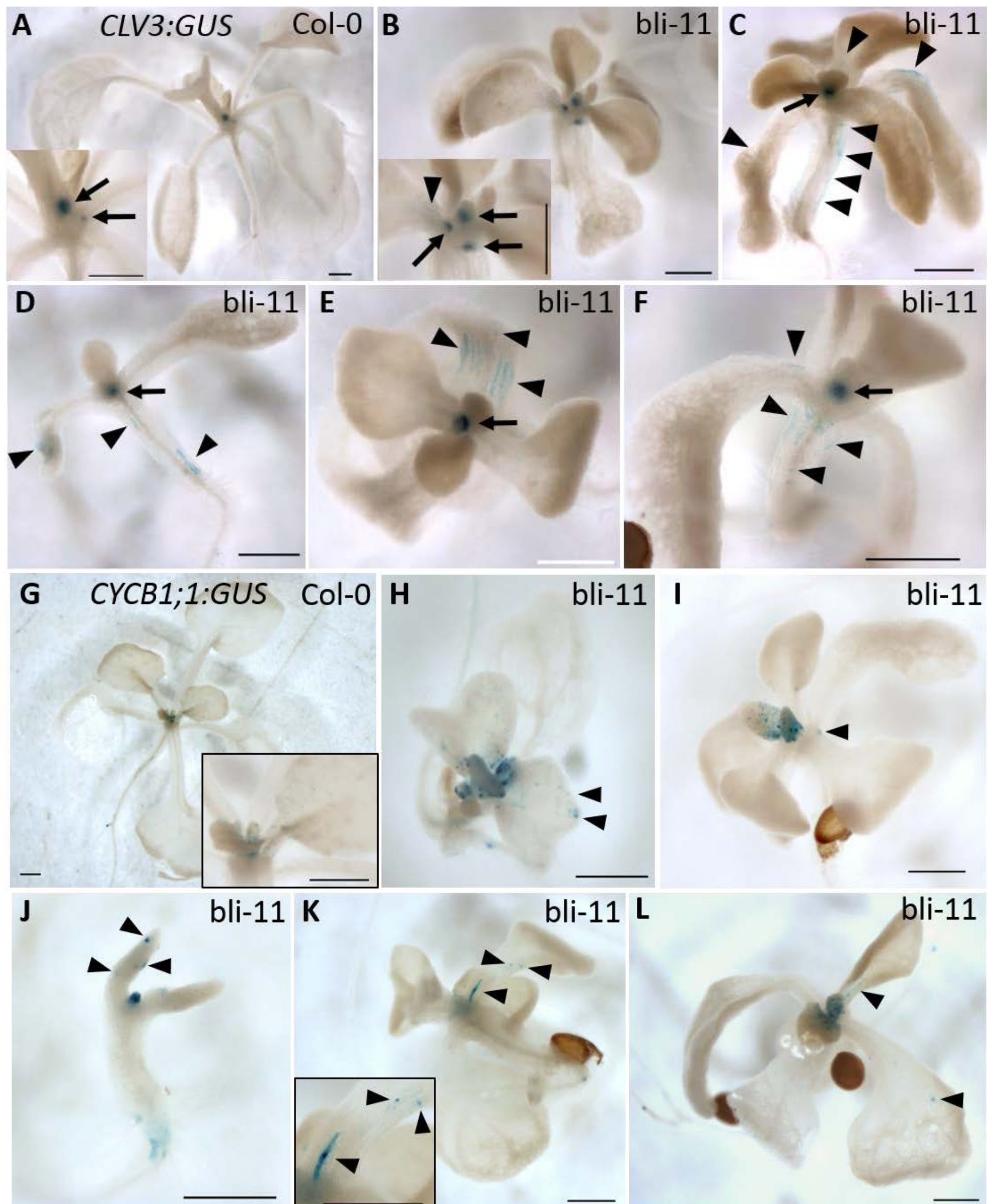

Figure 3: Expression of *CLV3:GUS* and *CYCB1;1:GUS* in *bli-11* mutants.
A) Col-0 seedlings showed SAM-specific *CLV3:GUS* expression. B-F) 43% of *bli-11* mutants showed an ectopic expression of *CLV3:GUS*, revealing a loss of cell identity. Arrows point to meristems (SAM and axillary) and arrowheads mark ectopic *CLV3:GUS* expression. G) Col-0 expressing *CYCB1;1:GUS*. H-L) 32% *bli-11* seedlings showed ectopic expression of *CYCB1;1:GUS*. Arrowheads mark ectopic *CYCB1;1:GUS* expression. Scale bars are 500 µm.

Stress-responsive genes are strongly up-regulated in *bli-1* mutants

In order to functionally characterize the mis-regulated genes in *bli-1*, we performed a GO-term analysis. We found enrichment of several GO-terms for stress-responses among the up-regulated genes in *bli-1*; a subset of these GO-terms is displayed in Table 4 (for full list of GO terms see Supplemental data 4). The most significantly enriched GO-terms for a specific form of stress were "response to endoplasmic reticulum stress" (GO-ID: 0034976) and "endoplasmic reticulum unfolded protein response" (GO-ID: 0030968). A GO-Slim analysis (Supplemental Figure 1) revealed a strong enrichment for the cellular component endoplasmic reticulum (ER), indicating a potential role of *BLI* in the ER-stress response/UPR (unfolded protein response). The GO-term "response to heat" (GO-ID: 0009408) was also enriched among up-regulated genes in *bli-1*. Interestingly, 3 out of 4 genes covered by this GO-term, namely *AtERDJ3A, BIP3* and *BIP1* (see Table 3), also act in the ER-stress response/UPR (Iwata et al., 2008; Kamauchi et al., 2005; Nagashima et al., 2011); hence, *BLI* might play a role in the regulation of stresses caused by accumulation of unfolded proteins (due to heat- or ER-stress).

Table 4: Selected GO-IDs enriched in up-regulated genes in *bli-1*.
Statistical significance was analyzed using the hypergeometric test with Benjamini-Hochberg correction; a p-value below 0.05 was considered as statistically significant.

GO-ID	term-name	p-value
GO:0006950	response to stress	9.75E-11
GO:0034976	response to endoplasmic reticulum stress	4.09E-11
GO:0030968	endoplasmic reticulum unfolded protein response	0.000484
GO:0009408	response to heat	0.005793
GO:0009414	response to water deprivation	0.012527
GO:0009737	response to abscisic acid stimulus	0.018293
GO:0009651	response to salt stress	0.040284

Table 5: Selected GO-IDs enriched in down-regulated genes in *bli-1*.
Statistical significance was analyzed using the hypergeometric test with Benjamini-Hochberg correction; a p-value below 0.05 was considered as statistically significant.

GO-ID	term-name	p-value
GO:0010374	stomatal complex development	6,75E-06
GO:0048367	shoot development	0,000113
GO:0048366	leaf development	0,000372
GO:0008544	epidermis development	0.000588
GO:0042335	cuticle development	0.003212
GO:0009409	response to cold	0.022874
GO:0009611	response to wounding	0.029012

Table 6: Mis-regulation of ABA-responsive genes in *bli-1* mutants.
In *bli-1* a significant number of ABA-responsive genes (Zeller et al., 2009) is mis-regulated. Also mis-regulated H3K27me3 target genes in *bli-1* are enriched for ABA responsive genes. *: total number of protein coding genes according to TAIR8 genome release. Statistical significance was analyzed using Chi square test with Yates correction; a p-value below 0.05 was considered as statistically significant.

	ABA-responsive genes	Total no. genes	percentage of ABA-responsive genes	Chi square test (p-value)
genome wide (Zeller et al., 2009)	2197	27235*	8.07	
***bli-1* up+down**	98	536	18.28	<0.0001
***bli-1* up**	55	292	18.84	<0.0001
***bli-1* down**	43	244	17.62	<0.0001
***bli-1* H3K27me3 target genes**	47	208	22.60	<0.0001

The “response to abscisic acid stimulus” (GO-ID: 0009737) was also significantly enriched among *bli-1* up-regulated genes. The phytohormone abscisic acid (ABA) promotes seed dormancy and desiccation tolerance and regulates embryo and seed development. In adult plants ABA regulates general growth and reproduction and is induced by abiotic stresses, such as drought, high salinity, and cold, and hence considered a “stress hormone” (reviewed in Tuteja, 2007). In *bli-1* seedlings we found a significant mis-regulation of ABA-responsive genes (Zeller et al., 2009) (Table 6, full list in Supplemental data 5). Additionally, a significant number ABA-responsive genes is also regulated by H3K27me3. This suggests an important function for BLI in regulating ABA-responsive PcG target genes. Interestingly, among the 18 commonly up-regulated genes in *bli-1* and *clf-28* mutants, 7 were regulated by ABA. Among mis-regulated ABA-responsive genes we did not detect key regulators of ABA biosynthesis or catabolism, or ABA reception or transport. This indicates that down-stream processes of ABA signaling, possibly genes transcriptionally regulated by ABA signaling, are affected in *bli-1*. As ABA regulates responses to drought stress and high salinity, it is consistent that the GO-terms “response to water deprivation” (GO-ID: 0009414) and “response to salt stress” (GO-ID: 0009651) were also significantly enriched among *bli-1* up-regulated genes. A detailed analysis of the genes belonging to the GO-term “response to water deprivation” revealed that most of these genes are directly regulated by ABA and targeted by H3K27me3. Taken together, up-regulation of genes in *bli* mutants which are regulated in “response to abscisic acid stimulus”, “response to water deprivation” and “response to salt stress” indicates a role of *BLI* in ABA-dependent gene regulation.

GO-term analysis of down-regulated genes in *bli-1* revealed strong enrichment of developmental processes, such as “stomatal complex development” (GO-ID:0010374), “shoot

development" (GO-ID:0048367), and "leaf development" (GO-ID:0048366) (Table 5). Our previous study indeed showed affected shoot and leaf development in *bli-1* (Schatlowski et al., 2010); also stomatal complex patterning is affected in *bli-1* (Supplemental Figure 5). Moreover, we previously showed that epidermis and cuticle development are affected in *bli-1*, resulting in gaps in the epidermis (Schatlowski et al., 2010). Consistent with this observation, we found the GO-terms "epidermis development" (GO-ID: 0008544), "cuticle development" (GO-ID: 0042335), and "response to wounding" (GO-ID: 0009611) among the down-regulated genes in *bli-1* (Table 5). A study by Purdy et al. (2010) showed that the induction of cold stress-responsive genes was impaired in *bli* mutants exposed to prolonged cold. Conclusively, the GO-term "response to cold" (GO-ID: 0009409) was enriched among down-regulated genes in *bli-1* revealing that, even under ambient temperatures, the expression of cold regulated genes is affected. Taken together, the GO term analysis of up- and down-regulated genes in *bli-1* strongly indicates a role for BLI in repression of stress-responsive genes and promotion of genes involved in developmental control.

To confirm that BLI plays an important role in the regulation of stress responses we performed principal component analysis (PCA) on the expression patterns of *bli-1* and responses to cold, drought, wounding (Kilian et al., 2007), and ER-stress (Nagashima et al., 2011) (Figure 4). *bli-1* clustered strongly with responses to prolonged drought, wounding, and with ER-stress. PC1 separated *bli-1* from prolonged cold stress (>3h) as well as short-term wounding responses. PC2 separated *bli-1* from short-term responses to cold, drought, and wounding. PC1 and PC2 could explain about 25% and 13% of the observed variance in the data, respectively, hence showing that those PCs were relevant for revealing differences between samples/treatments. The results of our PCA further indicate that BLI is an important regulator of several stress responses.

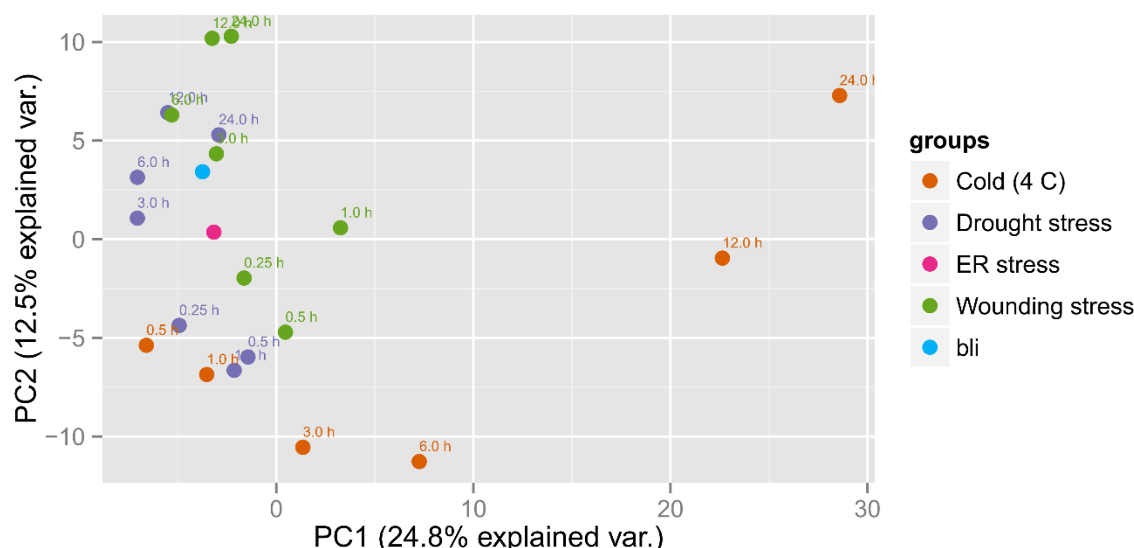

Figure 4: Principal component analysis (PCA) of *bli-1* mutants and several stress responses.
Results from the *bli-1* microarray were compared to responses to cold, drought, and wounding (Kilian et al., 2007) as well as ER-stress (Nagashima et al., 2011).

Finally, we tested if BLI is also involved in biotic stress responses and compared mis-regulated genes in *bli-1* with genes up-regulated by systemic acquired resistance (SAR) (Gruner et al., 2013). We found that a high number of genes up-regulated by SAR was also up-regulated in *bli-1* (Table 7). Interestingly, out of the 56 genes commonly up-regulated in *bli-1* and by SAR, 23 are also up-regulated in response to ER-stress. This fits to the observation that stress responses in plants are interconnected, and that a regulator of one stress can regulate several linked pathways or commonly regulated genes.

Table 7: Comparison of genes mis-regulated in *bli-1* and up-regulated by SAR.
A significant number of genes up-regulated in *bli-1* was also up-regulated by SAR (Gruner et al., 2013). *: total number of protein coding genes according to TAIR8 genome release. Statistical significance was analyzed using Chi square test with Yates correction; a p-value below 0.05 was considered as statistically significant.

	SAR (up)	Total no. genes	Percentage of SAR genes	Chi square test (p-value)
genome wide (Gruner et al., 2013)	547	27235*	2.01	
***bli-1* up**	56	292	19.18	<0.0001
***bli-1* down**	2	244	0.82	0.2840

bli mutants are hypersensitive to drought stress

The GO-term analysis and PCA strongly indicated that *BLI* plays an important role in several stress responses. In a previous study *BLI* was identified as a positive regulator of cold stress responses (Purdy et al., 2010). Because *bli-1* clustered strongly with long term drought stress responses in the PCA, we wanted to analyze the ability of *bli* mutants to cope with drought. For that purpose we subjected two strong *bli* mutants, *bli-1* and *bli-11*, to different periods of drought (Table 8) (for experimental setup see Supplemental Figure 4). The stress treatment

revealed that both *bli* mutants were hypersensitive to drought (Table 8 and Figure 5). Both complemented lines were able to rescue the drought-sensitive *bli* phenotype under the tested conditions, although the *bli-1/BLI:BLI-GFP* line showed a mild drought sensitivity after 0.5 and 1 h of drought stress, suggesting only partial complementation. These results show that loss of *BLI* reduces the ability of *bli* mutants to survive under drought stress conditions. Importantly, the gaps in the *bli-1* epidermis probably contribute to its drought stress sensitivity by elevating water loss.

Table 8: Survival of *bli* mutants and complemented lines after different periods of drought stress.
Five day old seedlings underwent 0h, 0.5h, 1h, and 2h of drought stress (see Supplemental Figure 4 for experimental setup) and were scored for survival 5 days after stress treatment. Four independent experiments with each two biological replicates were combined here. Ratios of all *bli* mutants were compared to the wild type. *: Ratios of complemented lines were compared to the respective mutant, to test the complementation ability. Statistical significance was analyzed using fishers exact test; a p-value equal to or below 0.05 was considered as statistically significant.

Exposure to drought (h)	0	0.5			1			2		
genotype	viable	viable	dead	fishers exact test p-value	viable	dead	fishers exact test p-value	viable	dead	fishers exact test p-value
Col-0	398	394	1		198	143		83	275	
bli-1	283	242	45	0.0001	73	192	0.0001	7	285	0.0001
bli-1/BLI:BLI-GFP	314	320	18	0.0001*	147	180	0.0001*	54	264	0.0001*
bli-11	231	126	47	0.0001	22	148	0.0001	5	150	0.0001
bli-11/BLI:BLI-GFP	349	340	5	0.0001*	170	150	0.0001*	137	207	0.0001*

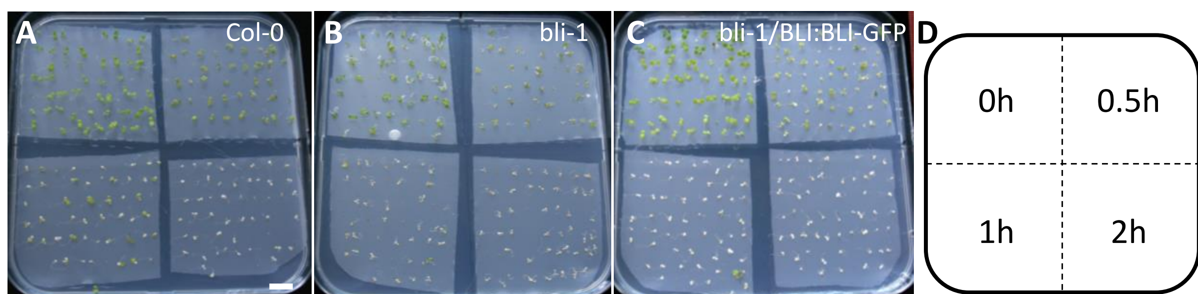

Figure 5: Drought stress treatment of *bli* mutants and complemented lines.
Five day old seedlings underwent 0h, 0.5h, 1h, and 2h of drought stress and were scored for survival 5 days after stress treatment. Survival of all genotypes was strongly reduced with increasing duration of drought stress treatment. Scale bar is 1 cm.

Discussion

Regulation of PcG target genes by BLI

We previously identified BLI as an interactor of the PRC2 methyltransferase CLF (Schatlowski et al., 2010). To further dissect the role of BLI in PcG-mediated gene regulation, we analyzed the transcriptome of *bli-1* seedlings and found a significant overlap of genes regulated by BLI and CLF. However, a high number of genes was not co-regulated by BLI and CLF, possibly because BLI has PcG-independent functions, or the function of CLF is masked by its partial

redundancy with SWN. To account for the latter, the overlap of genes regulated by BLI and CLF/SWN was analyzed, revealing a stronger co-regulation of genes by BLI and CLF/SWN as for CLF alone. This result indicates that BLI plays an important role in regulating a subset of genes targeted by PRC2 containing CLF or SWN. Importantly, transcriptional profiling of *bli-1* revealed a significant mis-regulation of PcG target genes, but no reduction or loss of H3K27me3 levels at these loci (Figure 2 A). Silencing of PcG target genes is not only dependent on PRC2 but also on PRC1, and other PcG proteins. The levels of H3K27me3 are affected in all analyzed PRC1 mutants but not at all PcG target genes (Calonje et al., 2008; Derkacheva et al., 2013; Wang et al., 2016; Yang et al., 2013). The PRC1 protein LHP1 can bind H3K27me3 via its chromodomain (Exner et al., 2009) and was shown to be important for maintenance of H3K27me3 after DNA replication, by interaction with the PRC2 protein MSI1 (Derkacheva et al., 2013). Importantly, the *lhp1* mutant only shows a mild phenotype (Turck et al., 2007), indicating that other mechanisms might be necessary for H3K27me3 maintenance after DNA replication. Interestingly, our previous study revealed that *bli-1* and *lhp1* genetically interact (Schatlowski et al., 2010). EMF1 is an interactor of MSI1 (Calonje et al., 2008) and like PRC2 mutants, *emf1* mutants show reduced H3K27me3 levels, but only at a subset of PRC2 target genes such as *AG* but not at *FUS3* (Calonje et al., 2008; Kim et al., 2012; Yang et al., 2013). Consistently, genome-wide EMF1 binding correlates with H3K27me3 (Kim et al., 2012). In *atbmi1a/b/c* triple mutants H3K27me3 levels at embryo developmental genes were reduced and were increased at meristem identity genes and flower developmental genes (Yang et al., 2013). Interestingly, the levels of H2Aub were increased in *clf swn* double mutants, hence, together with the increase of H3K27me3 in *atbmi1a/b/c,* demonstrating that PRC1 and PRC2 can partially balance each other's loss (Yang et al., 2013). The fact that levels of H3K27me3 are neither decreased nor increased in *bli-1* suggests that BLI is i) not involved in PRC2 recruitment, like LHP1 or EMF1, or ii) in H3K27me3 maintenance, like LHP1, and iii) likely has no PRC1 (*AtBMI1*) related function since H3K27me3 levels are not increased. Our results hence indicate that BLI most likely regulates PcG target gene expression downstream of, or in parallel to, PRC2. Additionally, BLI also represses genes independently of the PcG system.

As the action of PcG proteins is counteracted by Trithorax group proteins, we also tested H3K4me3 coverage of several up-regulated PcG target genes in *bli-1* mutants. Our analysis indicated that BLI is at least partially responsible for prevention of gain or increase of H3K4me3 at certain PcG target genes, such as at the ER-stress-responsive *SEC31A* locus (Figure 2 B). A recent study showed that during drought stress treatment levels of H3K27me3 remained constant at PcG target genes, while H3K4me3 levels increased resulting in active

transcription (Liu et al., 2014). Therefore, BLI might restrict binding of TrxG proteins to certain PcG target genes to prevent switches from repressive to active chromatin states during normal growth or under stress conditions. Future analysis of direct target genes and interaction partners of BLI will reveal if BLI directly interacts with PRC1 or TrxG proteins to stably silence genes or to restrict their activation, respectively.

BLI regulates specific developmental pathways

The strong *clf swn* or *vrn2 emf2* double mutants cannot sustain cell fate decisions during development, and develop into a callus-like cell mass early during seedling development (Chanvivattana et al., 2004; Schubert et al., 2005). Blister-like structures on several organs of *bli-1* mutants indicate a loss of cell identity. Moreover, *bli-1* mutants show enhanced endoreduplication and fewer cells, indicating a role for BLI in cell division regulation or cell cycle regulation. The stem cell marker *CLV3* and cell division marker *CYCB1;1* showed small domains of ectopic expression in *bli* mutants (Figure 3). *CLV3* is a PcG target gene encoding a precursor of a small secreted peptide which regulates SAM size (Brand et al., 2000; Fletcher et al., 1999). Thus, regulation of *CLV3* expression is likely a PcG-dependent function of BLI. The non-PcG target *CYCB1;1* is highly expressed in the G2/M phase of the cell cycle due to binding of TCP20 to its promoter (Li et al., 2005). *TCP20* is not mis-regulated in *bli-1*, indicating a direct and PcG-independent function of BLI in regulation of *CYCB1;1* expression. The ectopic expression of *CLV3* and *CYCB1;1* in *bli-11* hence suggests that BLI is a negative regulator of differentiation by preventing ectopic meristematic activity and endoreduplication without cell division. Whether BLI directly regulates *CLV3* and *CYCB1;1* will require further analyses.

Role of BLI in abiotic stress responses

Transcriptional profiling of *bli-1* mutants revealed a strong enrichment of stress-responsive genes among up-regulated genes. We found that genes involved in response to ER-stress, drought, high salt, heat, and genes up-regulated by systemic acquired resistance (SAR) were up-regulated in *bli-1*, whereas responses to cold and wounding were enriched among down-regulated genes (Table 4, Table 5 and Table 7). A principal component analysis (PCA) showed that *bli-1* expression profiles clustered with responses to drought, ER-stress, wounding, and, to a lesser extent, cold (Figure 4). Stress responses are cost-intensive, require extensive protein production in order to compensate for the stress, and consume important resources of a plant, which are required for growth and reproduction. Under ambient conditions it is important for a plant to prevent cost-intensive stress responses. To achieve this, stress responses are only

induced in response to stress and are suppressed under non-stress conditions. Our and others analysis indicates that BLI functions in both, activation and repression of stress responses.

Previously, *BLI* was identified as a positive regulator of cold stress responses; *bli* mutants showed a higher sensitivity to cold and reduced expression of cold-stress-responsive genes (Purdy et al., 2010). Our results further indicate that *BLI* is required for the activation of cold-stress-responsive genes, which is in contrast to its repressive function in the PcG pathway. The reduced induction and expression of cold-stress-responsive genes in *bli* mutants can also have another reason: BLI might regulate an unknown repressor of cold stress responses. Loss of *BLI* would activate the repressor and hence cold-responsive genes could not be properly induced or expressed during cold or even under ambient conditions. Analysis of interaction partners and direct target genes during cold will reveal how BLI regulates cold-stress responses.

The responses to drought and heat are connected: the transcription factor DREB2A was shown to have dual function in responses to drought and heat (Sakuma et al., 2006). Additionally, the drought-stress-responsive transcription factor NAC019, which is one of the up-regulated PcG target genes in *bli-1*, was recently reported to be heat-stress-responsive (Sullivan et al., 2014). The same study also discovered that *BLI* expression is highly increased in response to heat-stress (Sullivan et al., 2014). This observation and our own data indicate that BLI is also required for the regulation of heat stress responses. Up-regulation of genes induced by drought and heat indicate that BLI negatively regulates these responses. BLI might repress cost-intensive responses to these forms of stress during non-stress conditions and loss of *BLI* would lead to an induction of these stress responses in the mutant. This could explain why *bli* mutants were hypersensitive to drought stress: if the mutant already suffers from cost-intensive stress responses, additional stress treatment would lead to an inability to further respond to this stress, ultimately killing the plant. Additionally, *BLI* could promote resistance to stress, thereby acting on both, stress prevention during normal growth and resistance to a given stress. As *bli-1* mutants show defects in the epidermis and cuticle, leading to fast water loss, this probably also contributes to the mutants' drought sensitivity. To understand how BLI regulates heat and drought stress responses, it will be important to determine which genes and proteins are bound by BLI during these forms of stress.

Responses to cold, drought, and high salt are mediated by abscisic acid (ABA)-dependent but also ABA-independent pathways. In *bli-1* the GO-term "response to abscisic acid stimulus" was enriched among up-regulated genes, and consistently a significant number of ABA-responsive genes was mis-regulated in *bli-1* (Table 6). Additionally, a significant number of

mis-regulated ABA-responsive genes is targeted by H3K27me3 (Table 6), indicating that BLI might be involved in the regulation of ABA-responsive PcG target genes.

The role of PcG proteins in stress responses is only emerging (reviewed in Kleinmanns and Schubert, 2014). PRC2 and PRC1 proteins were shown to be involved in the regulation of stress-responsive genes or regulators of stress responses. For example, the PRC1 RING-finger proteins AtBMI1a and AtRING1b, also known as DREB2A-INTERACTING PROTEIN 2 (DRIP2) and DRIP1, respectively, are important negative regulators of drought-responsive gene expression by targeting DREB2A to 26S proteasome-mediated proteolysis (Qin et al., 2008). However, the role of AtBMI1a and AtRING1b in PcG-dependent silencing of drought-stress-responsive genes has not been resolved. EMF1 and EMF2 repress several categories of stress-induced genes such as cold-stress induced *COR15A* (Kim et al., 2010). Under non-stress conditions EMF1 directly binds to genes involved in biotic and abiotic stress, and these binding sites largely overlap with H3K27me3 sites (Kim et al., 2012). However, target gene binding of EMF1 and EMF2 under stress conditions was not yet resolved. MSI1 was shown to be a negative regulator of drought stress responses; the *msi1* co-suppressed mutant *msi1-cs* was reported to be more resistant to drought stress (Alexandre et al., 2009). Recently, a study revealed that MSI1 functions in a histone deacetylase complex to fine-tune ABA signaling and that loss of MSI1 led to an increased tolerance to salt stress (Mehdi et al., 2015). In the study by Mehdi et al. (2015) it was shown that MSI1 binds to chromatin of ABA receptor genes *PYL4*, *PYL5*, *PYL6* and that loss of MSI1 decreased levels of H3K9 acetylation at those loci. The level of H3K27me3 were not analyzed in the studies by Alexandre et al. (2009) and Mehdi et al. (2015), therefore it remains unclear if the PcG function of MSI1 plays a role in the regulation of stress-responsive genes. In contrast to *msi1-cs*, *clf* mutants showed a reduced resistance to drought (Liu et al., 2014). Interestingly, ABA levels were reduced during normal growth and during stress treatment in *clf* mutants (Liu et al., 2014). This indicates that during drought stress ABA-responsive genes might not be properly induced in the *clf* background, hence leading to reduced drought stress tolerance. Since genes involved in ABA biosynthesis or catabolism, or ABA reception or transport were not mis-regulated in *bli-1*, the reduced drought tolerance is likely due to a different mechanism than in *clf*. However, CLF and BLI are both necessary to cope with drought stress, and probably regulate certain ABA-responsive PcG target genes together.

In summary, our transcriptional profiling revealed that BLI regulates a subset of PcG target genes. Since H3K27me3 levels were not altered in *bli-1* mutants, BLI likely acts downstream of, or together with PRC2 in gene silencing. Moreover, we identified BLI as a regulator of

several stress responses, probably in a PcG-dependent manner. Therefore, BLI may be a key protein in connecting chromatin-mediated integration of stress responses, a process that is not well understood in plants. Analysis of BLI target genes and interaction partners under ambient and stress conditions will reveal which role BLI plays in PcG-dependent and -independent regulation of stress-responsive genes.

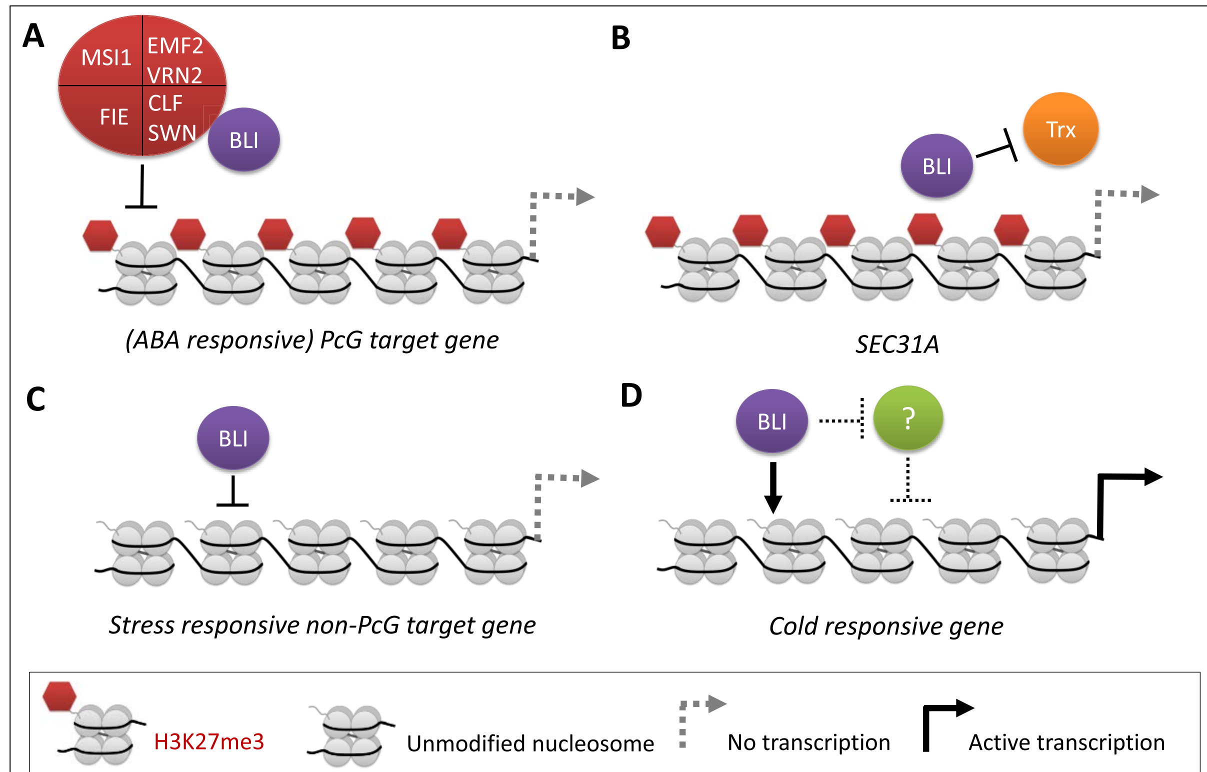

Figure 6: Regulation of gene expression by BLI.
A) BLI silences (ABA-responsive) PcG target genes, likely in parallel, or downstream of, PRC2. B) BLI likely prevents activation of certain PcG target genes by TrxG proteins. C) BLI represses stress-responsive non-PcG target genes under normal growth conditions. D) During cold stress BLI activates gene expression of cold-responsive genes, such as COR15A (Purdy et al., 2010), or represses an unknown repressor (indicated as '?') of cold-responsive genes. Whether BLI directly binds to stress responsive genes will be analyzed in the future.

Material and Methods

Plant material and growth conditions

Seeds of Columbia-0 (Col-0, N1092), *bli-1* (SAIL_107_D04, N805222), *bli-11* (GABI-Kat_663H12), *clf-28* (SALK_139371, N639371), *bli-1*/BLI:BLI-GFP (supplemental material and methods), and *bli-11*/BLI:BLI-GFP (supplemental material and methods) were sterilized (10 min 70% Ethanol supplemented with 0.05% Triton X-100, 10 min 96% Ethanol) and sown on ½ MS germination medium (half-strength Murashige and Skoog medium supplemented with 0.5% sucrose, 0.05% MES, and 0.8 % plant agar). Seeds were stratified for two days at 4°C and grown under long day conditions, (8/16 h dark/light rhythm at 20 °C). *bli-1* and *bli-11* seeds showed a germination delay of two days (Schatlowski et al., 2010). Therefore, when directly

compared, these two genotypes were sown two days earlier than all other genotypes, stratified for two days at 4°C and then transferred to the respective growth condition. For GUS staining, plants were grown for 14 days on ½ MS under long day conditions.

Microarray analysis

Seeds for microarray experiments were sterilized (5 min 70% Ethanol supplemented with 0.05% Triton X-100, 5 min 96% Ethanol) and sown on ½ MS. Seeds were stratified for two days at 4°C, grown under continuous light conditions for 12 days, and then harvested. RNA from whole seedlings was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden), resuspended in 30 µl RNAse-free water and treated with DNase (Fermentas). RNA quality was determined using a Bioanalyzer eukaryote total RNA nano chip (in cooperation with BMFZ, HHU Düsseldorf). RNA samples were processed by imaGenes GmbH (Berlin) with Agilent technologies using Arabidopsis 44k single colour arrays. The microarray was analyzed using background correction and quantile normalization of the limma package in the R environment (Ritchie et al., 2015; R Core Team, 2015). Differential expression was estimated using the empirical Bayes statistics implemented in limma (Ritchie et al., 2015; Smyth, 2004). P-values were adjusted for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Genes with a fold-change equal to or higher than 1.5, and with a p-value below 0.05, were included in further analyses.

bli-1 mis-regulated genes were compared to indicated gene sets using VirtualPlant 1.3 (Katari et al., 2010). For GO term analysis we used the online resource GOToolbox (http://genome.crg.es/GOToolBox/) and hypergeometric distribution with Benjamini-Hochberg correction for statistical analysis and p-value determination. For GOSlim analysis we used the online resource at "The Arabidopsis Information Resource" website (http://www.arabidopsis.org/tools/bulk/go/index.jsp) and statistically analyzed the data by Chi square test with Yates correction.

Chromatin immunoprecipitation (ChIP)

Plants were grown for 14 days on 1/2 MS under long-day conditions. 0.3-1 mg of seedlings were crosslinked using 1% FA fixation solution (10 mM Tris pH 7.5, 10 mM EDTA, 100 mM NaCl, 0.1% Triton X-100, 1% Formaldehyde) for 20 min under vacuum on ice. 2 M glycine was added to a final concentration of 0.125 M to stop the crosslink reaction. Samples were rinsed with ice-cold water to remove the fixation solution and frozen in liquid nitrogen.

20 µl Protein A coupled beads (Thermo Fisher Scientific) per sample were washed 3x with ChIP dilution buffer (1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH8, 167 mM NaCl,

0.2 mM PEFABLOC), then 1 µg antibody (anti-H3K27me3, C15410195 Diagenode; anti-H3K4me3, C15410003 Diagenode; anti-igG, C15410206 Diagenode) per 20µl beads was added and the mix was incubated rotating 10-12 h at 4°C. Frozen samples were ground in liquid nitrogen to a fine powder. Then 30 ml of Extraction buffer 1 (0.4 M sucrose, 10 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 5 mM beta-mercapto ethanol, 0.2 mM PEFABLOC, 1:200 plant proteinase inhibitor cocktail (Sigma Aldrich), and 1mM EDTA) were added to the powder, samples were vortexed and incubated 5 min on ice. The solution was filtered twice through 1 layer of Miracloth (VWR) and centrifuged for 20 min at 5000 g at 4°C. Supernatant was removed and pellet was washed twice with 1 ml Extraction buffer 2 (0.25 M sucrose, 10 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 1% Triton X-100, 5 mM beta-mercapto ethanol, 0.2 mM PEFABLOC, 1:200 plant proteinase inhibitor cocktail, 1mM EDTA). Samples were re-suspended in 300 µl extraction buffer 3 (1.7 M sucrose, 10 mM Tris-HCl pH 8, 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM beta-mercapto ethanol, 0.2 mM PEFABLOC, 1:200 plant proteinase inhibitor cocktail, 1mM EDTA), layered on 300 µl of extraction buffer 3 (sucrose gradient), and centrifuged for 1h at 16,000 g at 4°C. The pellet was re-suspended in 300 µl nuclear lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, 0.2 mM PEFABLOC, 1:200 plant proteinase inhibitor cocktail) and samples were sonicated 10-12 x (30 sec on, 60 sec off). Nuclear debris were removed by centrifugation for 5 min at 12,000 g at 4°C. Antibody-coupled beads and the no-antibody control beads were washed 3x with ChIP dilution buffer. 100 µl of sample and 900 µl of ChIP dilution buffer were added to 20 µl of beads and incubated rotating 10-12 h at 4°C for IP. Beads were washed 2x each with low salt wash buffer (150 mM NaCl, 0.1 % SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8), high salt wash buffer (500 mM NaCl, 0.1 % SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8), LiCl wash buffer (0.25 mM LiCl, 1% Nonidet-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8) and 1x with TE buffer (10 mM Tris-HCl pH8, 1 mM EDTA). To elute chromatin from the beads, 500 µl of 65°C warm elution buffer (1% SDS, 0.1 M $NaHCO_3$) were added, and samples were incubated for 30 min at 65°C with gentle shaking. The eluate was reverse-crosslinked by adding 20 µl of 5 M NaCl and incubation for 6-12 h at 65°C. Proteins were removed by adding 1 µl Proteinase K (20 mg/ml, Thermo Fisher Scientific), 10µl 0.5 M EDTA, and 20µl 1 M Tris-HCl (pH 6.5), and incubation for 60 min at 45°C. DNA was recovered using Phenol/Chloroform. The DNA pellet was re-suspended in 50 µl dH_2O. For qPCR analysis 2 µl of a 1:10 dilution of the DNA samples were used.

qPCR was performed in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using KAPA SYBR FAST qPCR Master Mix in a 2-step PCR program (95°C 3:00 min, 40 x (95°C

0:05 min, 60°C 0:30 min)). Values for immunoprecipitation (IP) were compared to input samples (=%IP). To account for differences in IP efficiencies and depending on the analyzed modification, %IP values were normalized to the *FUSCA3* locus (AT3G26790, H3K27me3 ChIP), which carries H3K27me3 and is not expressed in wild type and *bli-1*, and *ACTIN7* (AT5G09810, H3K4me3 ChIP), which carries high levels of H3K4me3 and is strongly expressed in wild type and *bli-1*.

Principal component analysis (PCA)

Expression profiles of responses to abiotic stress were obtained from the AtGenExpress dataset (Kilian et al., 2007) and a recent study on ER-stress induced by the drug tunicamycin (Nagashima et al., 2011). The dataset of Nagashima et al. (2011) was evaluated using the robust multi-array average (RMA) expression measure (Wu and Irizarry); the AtGenExpress data was provided in preprocessed form. Comparable distributions of gene expression were produced by quantile normalization, and replicates were averaged to compute fold changes. In the cases of stress treatment, expression was normalized against control, while the data on *bli-1* was normalized against the wild type. We performed principal component analysis on the log2-tranformed fold changes in gene expression using the prcomp() function of the stat package in R (R Core Team, 2015).

Stress experiments

For drought stress experiments petri dishes containing GM (half-strength Murashige and Skoog medium supplemented with 0.5% sucrose and 0.05% MES; hereafter: 1/2 MS) were covered with 4 separate membrane pieces (Sefar Nitex membrane 03-200/54, pore size: 200 µm/diameter) and sterile seeds were placed on top of each membrane (for visualization of experimental setup see Supplemental Figure 4). Seeds were stratified for 2 days at 4°C and grown under long day conditions (16/8 h light/dark). The membranes pore size of 200 µm/diameter ensured proper imbibition of seeds and a penetration by roots. Drought stress was applied 5 days after germination. Under a sterile bench the membranes with young seedlings were transferred to sterile, empty petri-dish lids. For the 0h control, membranes were lifted up and directly placed back on 1/2 MS to avoid possible artifacts caused by lifting up the membrane. Constant airflow in the sterile bench ensured that the seedlings placed on lids were exposed to drought. After 0, 0.5, 1, and 2 hours the membranes with seedlings were transferred back to 1/2 MS. After stress treatment, seedlings were grown for additional 5 days on 1/2 MS, then survival was scored.

GUS staining

Detection of ß-Glucuronidase (GUS) activity was performed according to Jefferson et al. (1987) with some modifications. Plants were fixed with 90% acetone for 30 min on ice and then washed for 20 min on ice with solution I (35 mM Na_2HPO_4, 13 mM NaH_2PO_4, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 1 mM EDTA, 500 µl Triton X-100 in 50 ml dH_2O). Solution I was replaced by GUS-staining solution (35 mM Na_2HPO_4, 13 mM NaH_2PO_4, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 500 µl Triton X-100, 5 mg X-Gluc in 50 ml dH_2O) and samples were incubated for 2-12h at 37°C. Samples were washed with dH20 and destained with 70% Ethanol. Plants were analysed and imaged using a stereomicroscope (Stemi 2000-C, Zeiss) equipped with AxioCam ICc1 (Zeiss).

Acknowledgements:

J.A.K. and D.S. are supported by a 'PLUS3' grant from the Boehringer Ingelheim Foundation.

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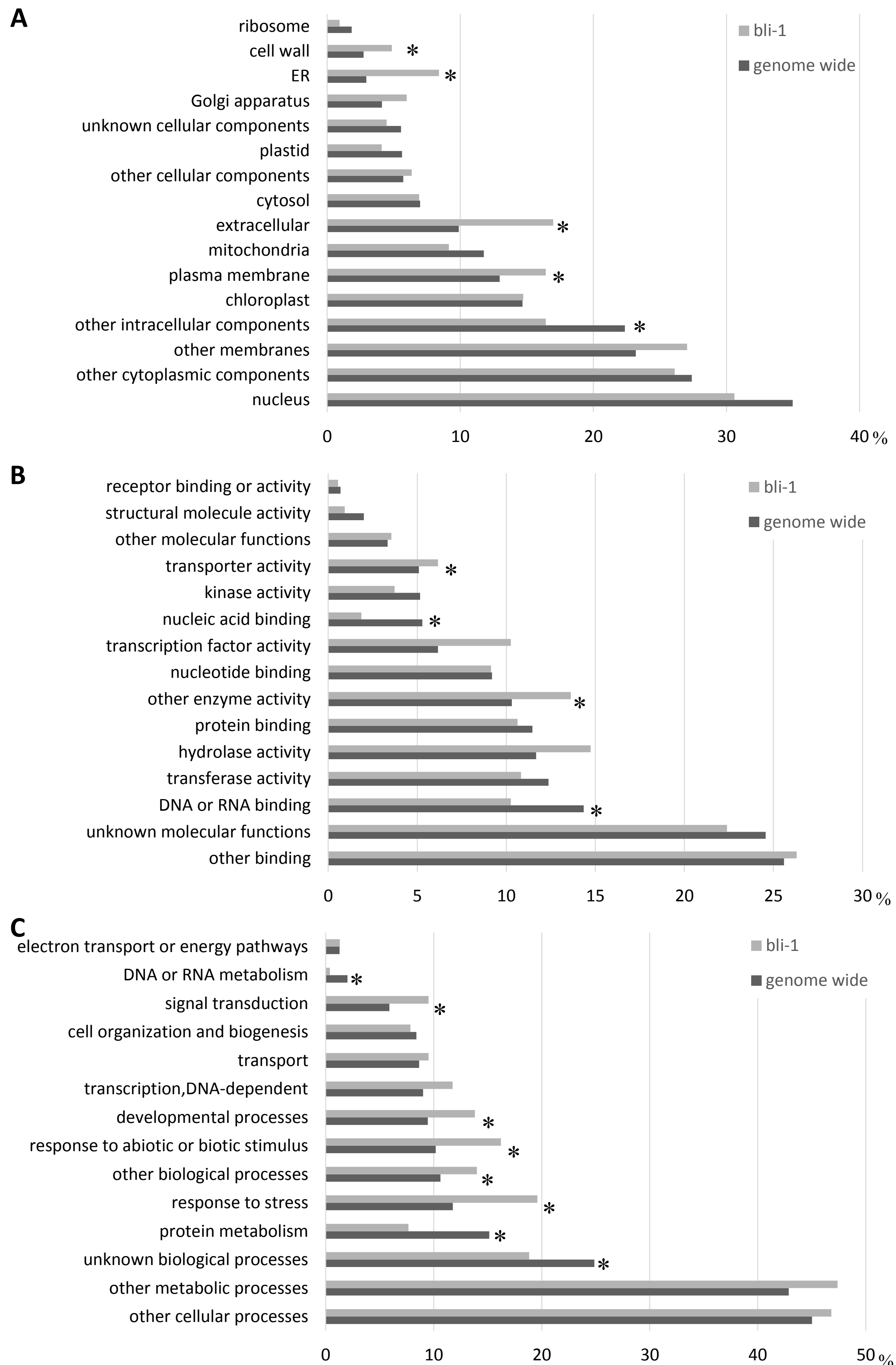
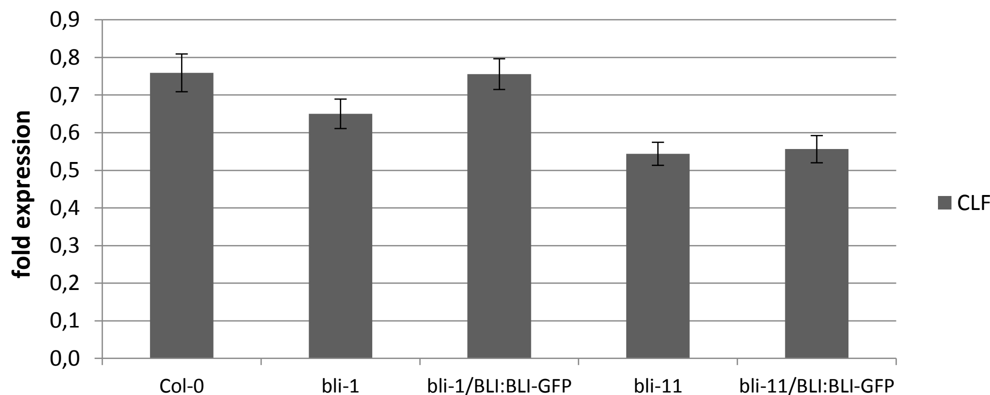
Supplemental Information



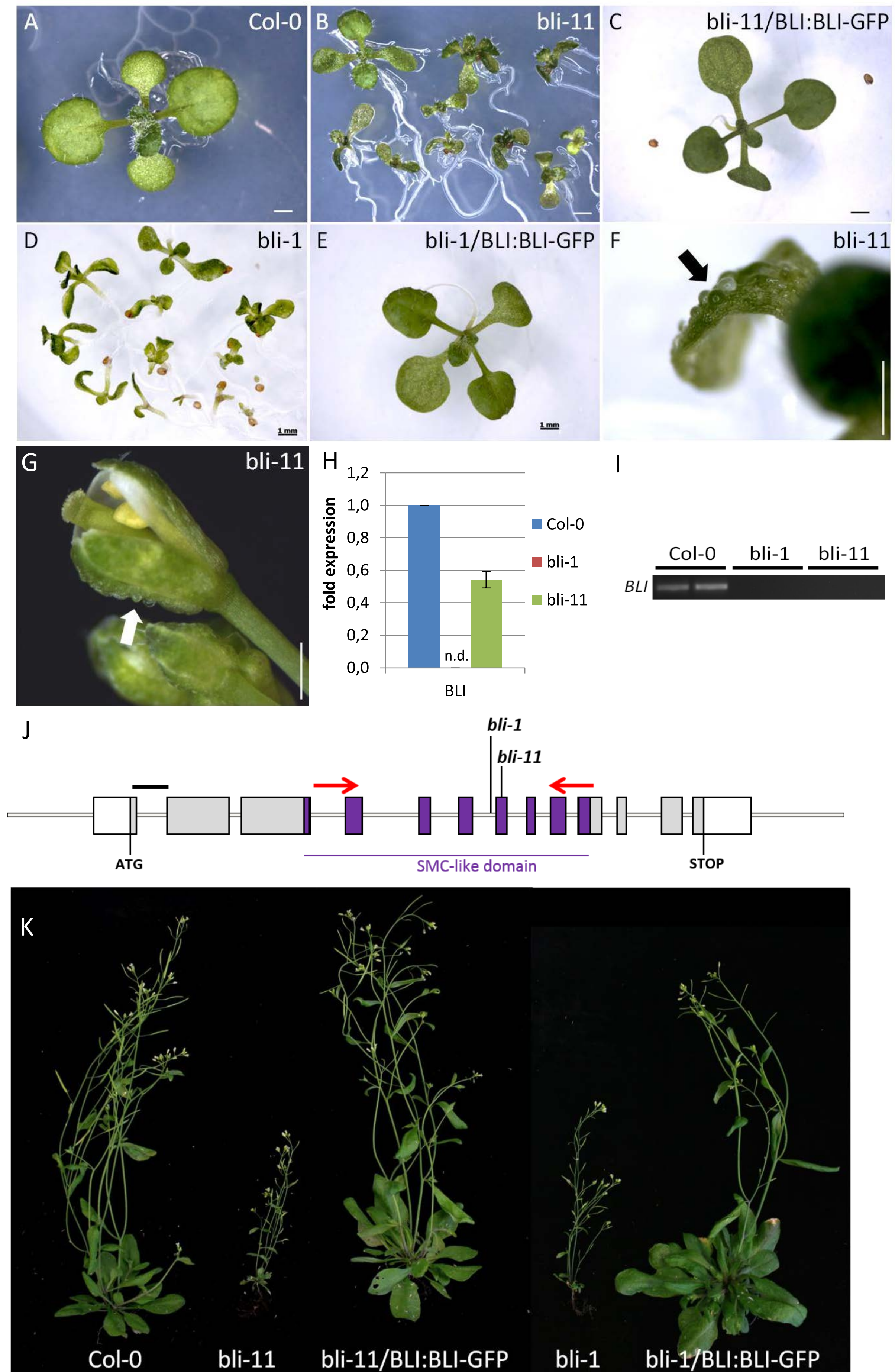
Supplemental Figure 1: GO Slim analysis of all up- and down-regulated genes in *bli-1* seedlings vs. genome wide.
A) GO Cellular Component. B) GO Molecular Function. C) GO Biological process. Asterisks indicate significant changes. Statistical significance was analysed using Chi square test with Yates correction; a p-value below 0.05 was considered as statistically significant.



Supplemental Figure 2: Expression of *CLF* in *bli* mutants and complemented lines.
CLF transcription is not changed in *bli* mutants or complemented lines compared to the wild type. Statistical significance was analysed using Student´s t-test; a p-value below 0.05 was considered as statistically significant.

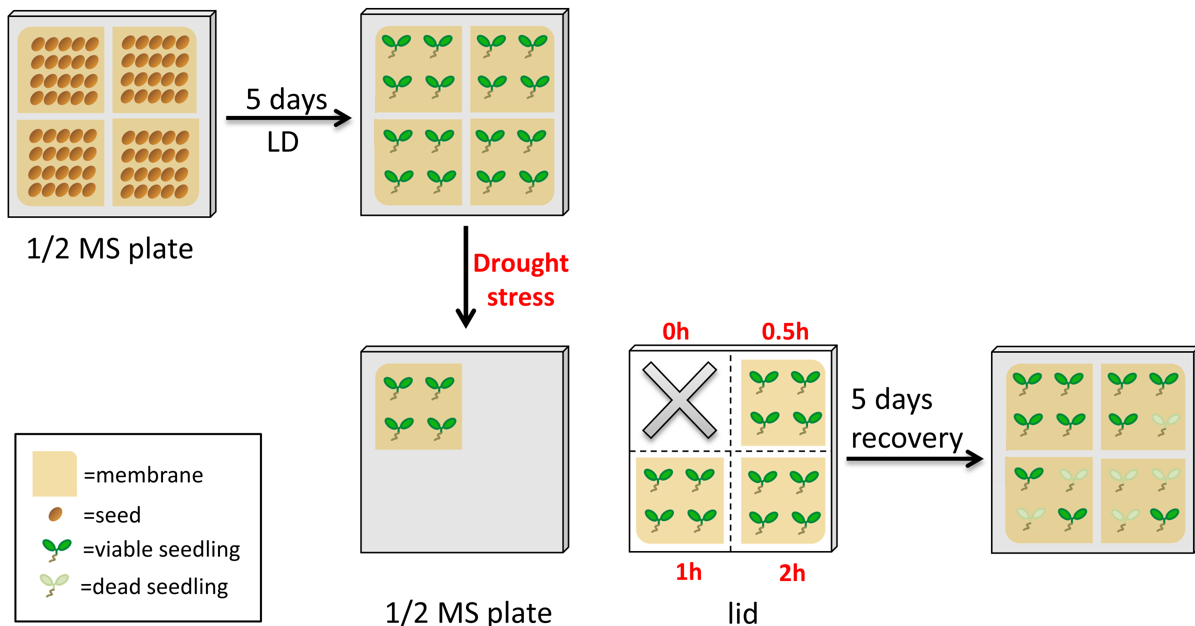
Characterization of the novel *bli-11* mutant

In this study, we characterized a novel *BLI* allele, *bli-11* (GABI-Kat_663H12). *bli-11* is the only available *BLI* allele comprising a T-DNA insertion in an exon (exon no. 7) (Supplemental Figure 3 J). Only *bli-1* and *bli-11* contain a T-DNA insertion in the highly conserved SMC-like domain, which is the domain important for interaction with the PRC2 member CURLY LEAF (CLF) (Schatlowski et al., 2010). Analysis of *BLI* transcript level in *bli* mutants*,* revealed a reduced transcription in *bli-11* and no transcript in *bli-1* (Supplemental Figure 3 H, J). Importantly, we were unable to detect a full length transcript containing the SMC-like domain in *bli-11* and *bli-1* (Supplemental Figure 3 I, J)*,* rendering *bli-11* a null or severe loss-of-function mutant. The *bli-11* mutant phenotypically resembles the *bli-1* mutant, showing a strong pleiotropic phenotype and blister-like structures on several organs (Supplemental Figure 3 F, G). Introduction of a genomic copy of BLI fused to GFP (*BLI:BLI-GFP*) could rescue the *bli-11* phenotype, showing that loss of *BLI* function was causing the observed *bli-1*-like phenotype of *bli-11* (Supplemental Figure 3 K). Because of the strong similarity of the severe loss-of-function mutant *bli-1* and *bli-11*, we included *bli-11* in our experiments.

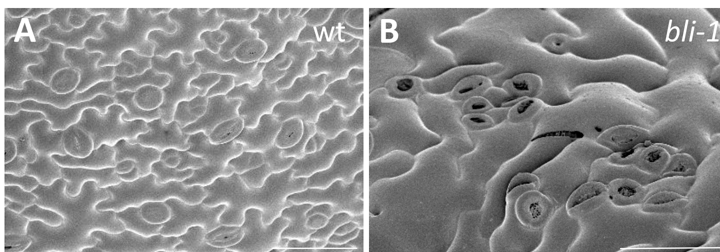


Supplemental Figure 3: Characterization of the novel *bli-11* mutant.

A-E) The *bli-11* mutant strongly resembles *bli-1*. Both mutants could be complemented by introduction of full-length genomic BLI fused to GFP (BLI:BLI-GFP) (K). F-G) *bli-11* mutants exhibit blister-like structures on cotyledons and flowers, a phenotype also observed in *bli-1*. H) The expression analysis revealed reduced *BLI* transcription in *bli-11* and no transcription in *bli-1* (n.d.: not detected; see horizontal black line in J for amplification site). Expression was normalized to *ACT2*, 2 biological replicates with 3 technical replicates each, ± SE of biol. replicates. I) No full-length transcript (region between red arrows in J) could not be detected in either *bli-11* or *bli-1,* indicating a non-functional *BLI* gene in both mutants (two biological replicates for each genotype are shown). J) *BLI* locus. Light grey and purple boxes indicate exons. Purple coloured boxes indicate exons coding for the conserved SMC-like domain, important for interaction with CLF. Horizontal black line indicates fragment amplified in H); red arrows show region amplified to test for full-length transcript in I). K) Adult *bli* mutants and the respective complemented lines. A *BLI:BLI-GFP* transgene could complement the *bli* phenotype.



Supplemental Figure 4: Drought stress treatment procedure.
Petri-dishes containing half-strength Murashige and Skoog medium supplemented with 0.5% sucrose (1/2 MS) were covered with Sefar Nitex membrane (03-200/54) with a pore size of 200µm/diameter. Sterile seeds were grown on top of this membrane under long day conditions (16/8 h light/dark). Drought stress was applied 5 days after germination. For drought stress, the membrane containing the young seedlings was placed in a sterile petri-dish, while constant air-flow, and transferred back on the initial ½ MS plate after 0, 0.5 , 1, and 2 hours. For the 0h control the membrane was only lifted up and directly placed back on 1/2 MS to avoid possible artefacts caused solely by lifting up the membrane/seedlings. Constant airflow in a sterile bench ensured that the seedlings on the lid were exposed to drought stress conditions. Seedlings were grown for additional 5 days on 1/2 MS, then survival was scored.



Supplemental Figure 5: Stomatal patterning in *bli-1*.
Stomatal patterning is affected in *bli-1* (B) compared to the wild type (A). Scale bars are 50 µm.

Supplemental Table 1: Oligonucleotides used for H3K27me3 and H3K4me3 ChIP qPCR.

gene name	ATG number	F	R
PI	AT5G20240	CCACATATCCTCTCCTCCATA	CCATTCCTCCTCTTTGAGAACG
SEP2	AT2G21970	TGTTTTTGATGCGTGAGGTT	CAAAGCTCTGTTGGCATCAA
SEP3	AT1G24260	GGGTTTCCAATTTTGGGTTT	GATGAATCCCATCCCCAAGT
AG	AT4G18960	TGGGTACTGAGAGGAAAGTGAGA	GGATCGTAGAAGGCAGACCA
BIP3	AT1G09080	GTGAGCTTGCGAAACGATCT	CCTCGAATCTTGCTCTCGTT
SEC31A	AT1G18830	TACAAGGAAGCAGTGGCTCA	CCCACAATTCTGTACCACCA
LTP2	AT2G38530	GCAACGGCGTTACTAACCTT	TTTAGCGGCAGATTGAAGGC
Threonyl-tRNA synthetase	AT1G17960	CTTCCGGCTTGCTTCAAACT	AGATCCCAACACCGCACTAT
UDP-Glycosyltransferase superfamily protein	AT3G55700	TTCAACCCCATGATCGAGCT	AGAAGGATCGGGGAAGTTGT
ACT7	AT5G09810	TAGTGAAAAATGGCCGATGG	CCATTCCAGTTCCATTGTCA
FUS3 (Kwon et al., 2009)	AT3G26790	GTGGCAAGTGTTGATCATGG	AGTTGGCACGTGGGAAATAG
SEP2-ATG	AT3G02310	TTTTGGGGTGAGGAAAGATG	CGCAGAGAACAGAAAGCTCA
SEP3 -ATG	AT1G24260	TGACGTTTGCAAAGAGAAGG	GCATGCTCGAACTACTGCAA
BIP3 (Song et al., 2015)	AT1G09080	CACGGTTCCAGCGTATTTCAAT	ATAAGCTATGGCAGCACCCGTT
SEC31A (Song et al., 2015)	AT1G18830	GAACTCGATTTTCAGTCCAA	TTGGATTCCATAAACCGATG

Supplemental Table 2: Oligonucleotides used for qRT-PCR analysis of *BLI* and *CLF* expression.

gene name	ATG number	F	R
BLI	AT3G23980	AGAGGGAACATTTCCCTCTG	GAAACTGCTCAAGCTTACGG
ACT7	AT5G09810	CCAGGAATTGCTGACCGTAT	GGTGCAACCACCTTGATCTT
CLF	AT2G23380	TTTCGATAACCTGTTCTGCC	GTCTCCCACTACCTTTCACC
PP2A-1 (Czechowski et al., 2005)	AT1G59830	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA

Supplemental methods

RNA isolation and qPCR

***BLI* transcript levels:** RNA from rosette leaves was extracted using RNeasy Plant Mini Kit (Qiagen), resuspended in 30 µl RNAse-free water, and treated with DNaseI (Fermentas). cDNA was synthesized from 1 µg RNA using SuperScriptII Reverse transcriptase Kit (Invitrogen) and Oligo(dT) oligonucleotides. The obtained cDNA was diluted 1:10 and 1 µl of this dilution was used for qRT-PCR. qPCR was performed in a Chromo4 real-time PCR machine (Bio-Rad) using MESA BLUE qPCR MasterMix Plus for SYBR® Assay (Eurogentech) in a 2-step PCR program (95°C 5min, 40 x (95°C 0:15 min, 60°C 1:00 min)). Expression levels were normalized to *ACTIN7* (AT5G09810).

***CLF* transcript levels:** RNA from 14-day old seedlings was extracted using innuSPEED Plant RNA Kit (Analytik Jena), resuspended in 30 µl RNAse-free water, and treated with DNaseI

(Fermentas). cDNA was synthesized from 1 µg RNA using RevertAid RT Reverse Transcription Kit (Thermo Scientific) and Oligo(dT) oligonucleotides. The obtained cDNA was diluted 1:10 and 2 µl of this dilution was used for qRT-PCR. qPCR was performed in a LightCycler 480 (Roche) using KAPA SYBR FAST qPCR Master Mix in a 2-step PCR program (95°C 5:00 min, 40 x (95°C 0:15 min, 60°C 0:30 min)). Expression levels were normalized to AT1G59830 (*PP2A-1*) (Czechowski et al., 2005).

Cloning of pGKGWG-gBLI

Genomic *BLI* (*gBLI*), containing the *BLI* coding region and 1.7 kb upstream of the transcriptional start site, was amplified from genomic DNA using oligonucleotides F: GGGGACAAGTTTGTACAAAAAAGCAGGCTGAACTGGCAATTCAGAATCGGG, R: GGGGACCACTTTGTACAAGAAAGCTGGGTGGAGAAGCTTGCTTGTCCTTCTTTTC, and introduced into pDONR201 (Invitrogen). *gBLI* was cloned into pGKGWG (Zhong et al., 2008) using GATEWAY technology (Thermo Fisher Scientific), according to the manufacturers´ instructions.

Plant transformation:

bli-1 and *bli-11* heterozygous mutants were transformed with pGKGWG-gBLI using the floral-dip method (Clough and Bent, 1998) and *Agrobacterium tumefaciens* strain GV3101 pMP90 (Koncz and Schell, 1986).

3.2 Localization and expression of GFP-CLF in *bli* mutants

In *bli-1* a high number of PcG target genes was mis-regulated while levels of H3K27me3 at these genes were not changed (manuscript I). Early experiments in the PRC1 *lhp1* mutant showed that H3K27me3 levels were not changed (Turck et al., 2007), like in *bli-1*. As *LHP1* is strongly expressed in proliferating cells, Derkacheva et al. (2013) induced lateral root outgrowth in *lhp1* mutants and used this tissue for analysis of H3K27me3 levels in the mutant. The authors could show that H3K27me3 levels were reduced in dividing tissues of *lhp1*, and proposed that LHP1 is important for recruitment of PRC2 to target genes after DNA replication (Derkacheva et al., 2013). As the *lhp1* mutant shows a mild phenotype (Turck et al., 2007), the proposed model of PRC2 recruitment by LHP1 is likely not the only mechanism for maintenance of H3K27me3 marks after DNA replication. Interestingly, *bli-1* and *lhp1* were shown to genetically interact (Schatlowski et al., 2010). To reveal if the PRC2 methyltransferase CLF is mis-regulated in *bli* mutants, probably in specific cell types, its sub-cellular localization in root cells was analyzed. Therefore, a *35S:GFP-CLF* construct (Schubert et al., 2006) was introduced into *bli-1* and *bli-11.* The localization, expression strength and protein levels of GFP-CLF were dissected by confocal laser scanning microscopy (Figure 3.1), quantitative RT-PCR, and immunoblots (Figure 3. 2).

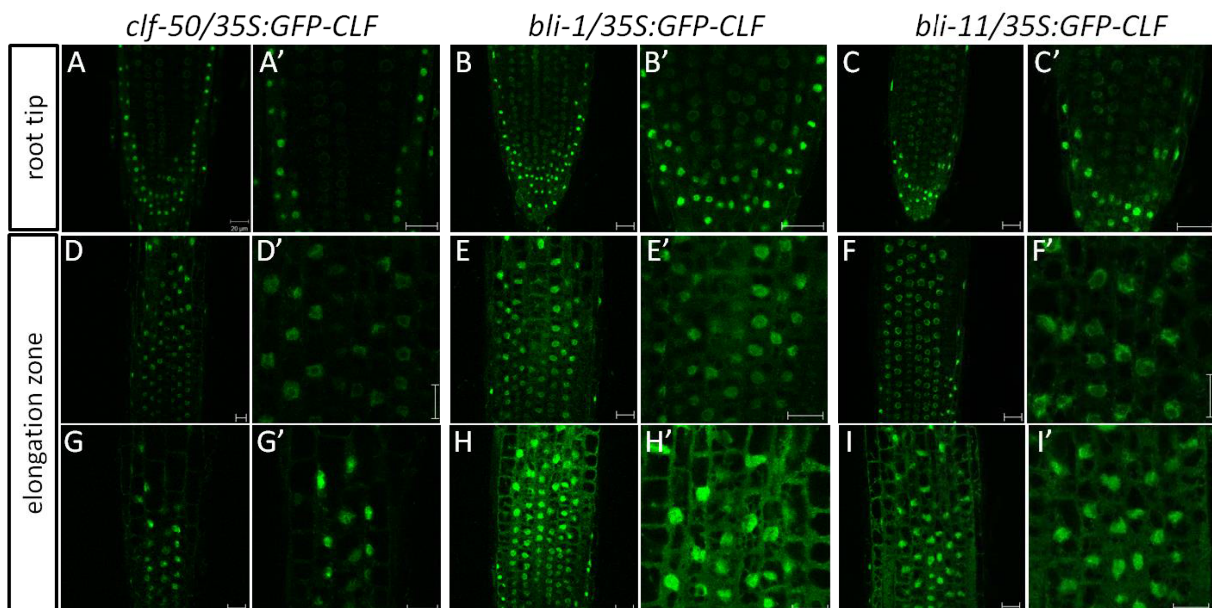


Figure 3. 1: Laser-Scanning confocal microscopy pictures of *35S:GFP-CLF* in *clf-50, bli-1* and *bli-11* mutants.
A-C) Pictures of root tips of *clf-50*, *bli-1* and *bli-11* expressing *35S:GFP-CLF* with magnifications in (A', B', C'). D-I) Cells of the root elongation zone of two independent *clf-50* (D, D', G, G'), *bli-1* (E, E', H, H'), and *bli-11* (F, F', I, I') plants expressing *35S:GFP-CLF*, and the respective magnifications ('). Confocal pictures were taken with same laser settings and have not been modified by quality enhancing tools, to show true expression strength of GFP-CLF in the respective mutants. Scale bars are 20 µm.

In *bli-1* and *bli-11* the fluorescence of GFP-CLF in the nuclei of cells of the root tip and elongation zone was stronger compared to *clf-50* (Figure 3. 1). GFP-CLF fluorescence in the

cytoplasm of root cell was also stronger in *bli-1* and *bli-11* compared to *clf-50*. This observation is in contrast to the expression level of (*GFP*-) *CLF* in these mutants (Figure 3. 2).

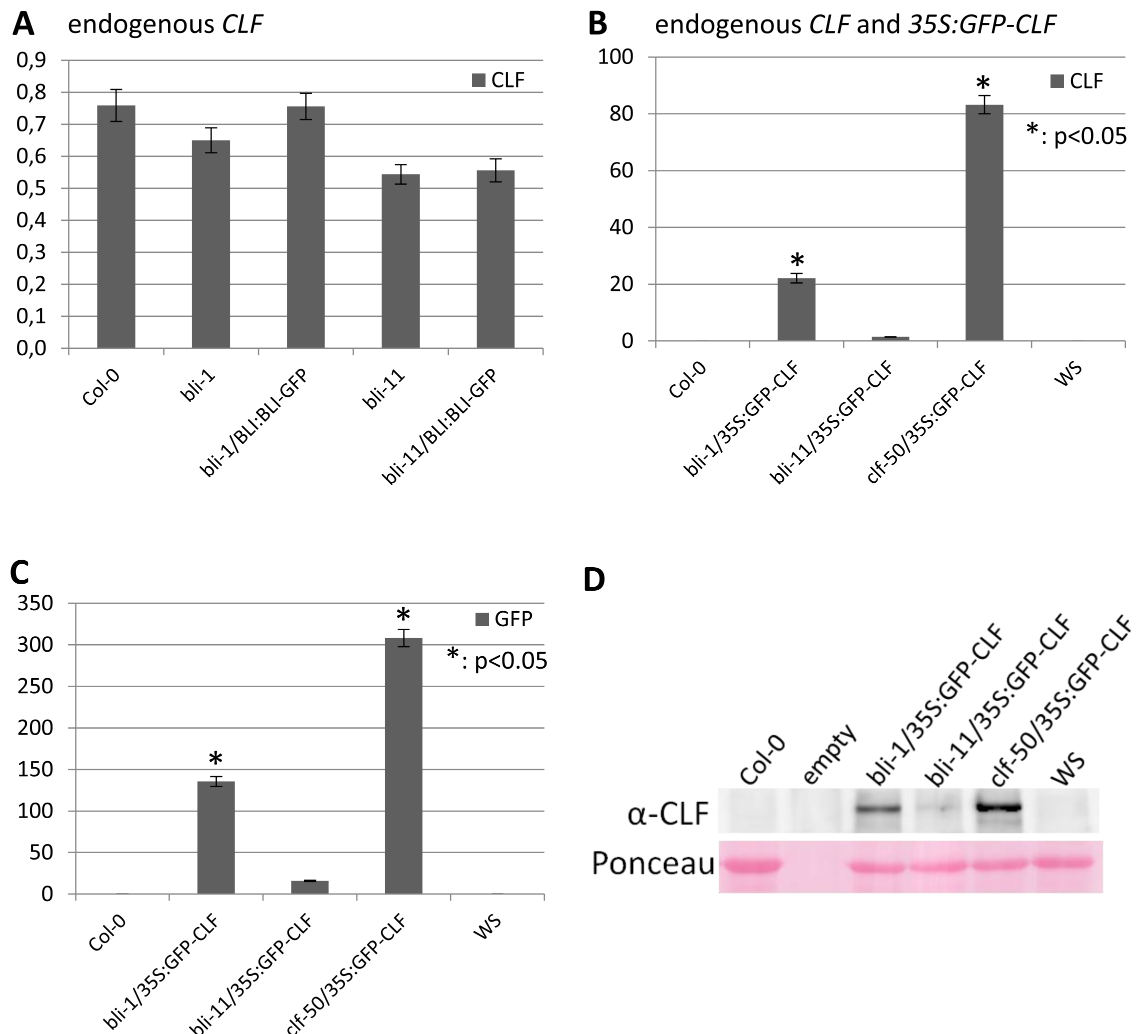


Figure 3. 2: Expression and protein levels of endogenous CLF and GFP-CLF in *bli* mutants.
A) Expression of endogenous *CLF* in *bli-1*, *bli-11* and the respective complemented line. B) Expression of endogenous *CLF* and transgenic *35S:GFP-CLF* in *bli-1*, *bli-11* and *clf-50* mutants. C) Expression levels of *GFP* in *bli-1*, *bli-11* and *clf-50* mutants expressing *35S:GFP-CLF*. Two independent quantitative RT-PCR experiments were performed with 3 technical replicates per genotype; Statistical significance was analysed using Student´s t-test, a p-value equal to or below 0.05 was considered as statistically significant. D) Immunoblot using whole plant extract and antibodies directed against the N-terminus of GFP-CLF. Ponceau S staining after protein detection was used as loading control. One experiment with one biological replicate per genotype was performed. Band intensities in (D) are consistent with expression levels in (B) and (C).

Endogenous *CLF* was not mis-regulated in *bli* mutants (Figure 3. 2 A). In contrast, *35S:GFP-CLF*-expressing *clf-50* and *bli-1,* but not *bli-11,* showed high expression levels of (transgenic) *CLF* (Figure 3. 2 B). The same pattern was observed for *GFP* expression in these mutants (Figure 3. 2 C). The high expression of transgenic *CLF* and *GFP* is consistent with preliminary immunoblot data (Figure 3. 2 D), showing high CLF protein levels in *clf-50* and *bli-1,* and weaker levels in *bli-11*. The high gene expression and protein levels of CLF in *clf-50* are inconsistent with the fluorescence intensity of GFP-CLF in this mutant. One explanation would

be that BLI negatively affects *35S:GFP-CLF* transgene expression, leading to strong *CLF* and *GFP* expression in the *bli-1* mutant. Another possibility is that BLI negatively regulates CLF protein stability and that the higher GFP signal is due to a lack of protein degradation in *bli* mutants. If BLI reduces CLF protein stability, this would probably be reflected by higher H3K27me3 levels in *bli* mutants, which is not the case. Therefore, it remains elusive how BLI affects CLF localization and stability.

3.2 Genome-wide levels of H3K4me3 are not changed in *bli* mutants

In manuscript I and manuscript II in this study, the levels of H3K4me3 of several genes in *bli* mutants were analyzed by ChIP. A few genes gained H3K4me3 in *bli* mutants, but H3K4me3 target genes were not enriched among the mis-regulated genes in *bli-1* (manuscript I, Table 2). To confirm that there are no significant genome-wide changes in H3K4me3 levels in *bli*, immunoblots using primary antibodies directed against H3 and H3K4me3 in combination with fluorescent secondary antibodies were used (Figure 3. 3 A). This analysis revealed no significant differences in genome-wide H3K4me3 levels in *bli-1*, *bli-11*, *clf-28*, and the complemented *bli/BLI:BLI-GFP* lines, in comparison to the wild type (Figure 3. 3 B).

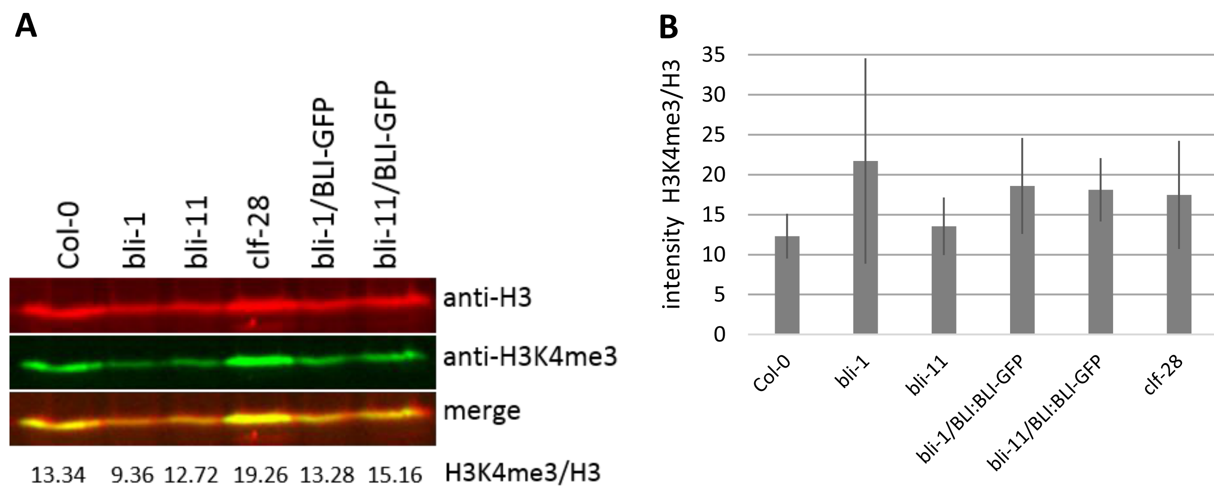


Figure 3. 3: Immunoblots detecting genome-wide H3K4me3 modifications.
A) Immunoblot detecting H3 (red) and H3K4me3 (green) levels in Col-0, *bli-1*, *bli-11*, *clf-28*, and the complemented *bli*/BLI:BLI-GFP lines. Please note that the different protein loading does not affect the experiment, as H3 and H3K4me3 are detected simultaneously on the same membrane. Ratios of H3/H3K4me3 of each sample are shown below the merged channels.
B) Levels of H3K4me3 in all tested mutant lines were not changed compared to the wild type. Three independent experiments with each 1 biological replicate are shown. Statistical significance was analyzed using Student´s t-test, a p-value below or equal to 0.05 was considered to be statistically significant.

3.3 Manuscript II

Endoplasmic reticulum (ER-) stress in plants can be caused by extensive gene expression during development, as well as by abiotic and biotic stress, leading to an accumulation of unfolded proteins in the ER lumen (Che et al., 2010; Deng et al., 2011; Gao et al., 2008; Liu et al., 2007b; Liu et al., 2011a; Moreno et al., 2012). In non-stress conditions, it is important that ER-stress is suppressed to prevent spurious degradation of proteins by the unfolded protein response (UPR), which is induced by mis- or unfolded proteins. So far no suppressor of ER-stress or the UPR has been identified in plants. The transcriptional profile of *bli-1* mutants showed a significant up-regulation of ER-stress-responsive genes (manuscript I). This indicates that BLI might be involved in the negative regulation of ER-stress responses. To understand how BLI regulates the ER-stress response I analyzed how *bli* mutants respond to ER-stress treatment and revealed a hypersensitivity. Previously, it was shown that ER-stress induced H3K4me3 at certain genes in *Arabidopsis* (Song et al., 2015). The analysis of H3K4me3 levels at certain genes in *bli* mutants revealed, that this histone modification is similarly elevated in non-stressed *bli-1* mutants as in ER-stressed wild type plants. These results strongly indicated that *BLI* is the first identified negative regulator of ER stress in plants.

The previous analysis of BLI subcellular localization revealed presence of BLI-GFP in nuclei and cytoplasmic ‘speckles’ (Purdy et al., 2010; Schatlowski et al., 2010). To determine the identity of these speckles, BLI-GFP was co-expressed with several fluorescent markers for cytoplasmic compartments such as the Golgi and the ER. This analysis showed that BLI-GFP partially colocalizes with the Golgi but strikingly not with the ER in *Arabidopsis*. To address which domains are responsible for the localization of BLI-GFP in nuclei, the subcellular localization of mutated BLI was analyzed. For this purpose BLIs nuclear import signal (NLS) and nuclear export signal (NES) were mutated. Additionally, a viral (SV40) NLS was added to reveal how constitutive nuclear localization of BLI affects plant growth. The nuclear localization of BLI appears to be tightly regulated as even the strong SV40 NLS was not always sufficient to confer nuclear localization of BLI-GFP.

These results are described in detail in Manuscript II “The nuclear and Golgi localized protein BLISTER is involved in the negative regulation of ER-stress responses in *Arabidopsis thaliana*”, in this work.

“The nuclear and Golgi localized protein BLISTER is involved in the negative regulation of ER-stress responses in *Arabidopsis thaliana*” – Manuscript II

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Keywords: Polycomb-group proteins, ER-stress, UPR, Tunicamycin, BLI, Golgi, H3K4me3

Author contributions:

J.A. Kleinmanns and D. Schubert designed the research. J.A. Kleinmanns performed all experiments. J.A. Kleinmanns wrote the manuscript, which was revised by DS.

Abstract

Abiotic and biotic stress can cause an accumulation of unfolded proteins in the endoplasmic reticulum (ER), leading to ER-stress. Unfolded proteins activate the unfolded protein response (UPR), which induces the expression of downstream genes such as chaperones and inhibitors of programmed cell death. BLISTER (BLI), a protein interacting with Polycomb group proteins, is involved in the regulation of abiotic stress responses. We previously revealed that genes responding to ER-stress are highly up-regulated in *bli* mutants. Here we dissected the role of BLI in ER-stress responses. Chromatin immunoprecipitation experiments revealed an increase in H3K4me3 levels at several ER-stress-responsive genes in the absence of BLI. Those genes were reported to acquire H3K4me3 in response to ER-stress. In response to ER-stress treatment, several ER-stress-responsive genes showed an even stronger up-regulation in *bli-1* mutants compared to the wild type, indicating that BLI is a negative regulator of ER-stress-responsive genes in *Arabidopsis thaliana*. We furthermore show that BLI-GFP fusion proteins localize to Golgi vesicles and p-bodies but not to the ER. Expression of truncated BLI-GFP in *Arabidopsis* indicates that the localization of BLI to Golgi vesicles and p-bodies is dependent on its C-terminal domain.

Introduction

In eukaryotic cells the endoplasmic reticulum (ER) is an important organelle for protein folding, modification and assembly. The ER contains several chaperones and foldases which help to properly fold proteins to prevent aggregation. During development, cells undergo substantial changes in their gene expression patterns. High levels of gene expression can cause an accumulation of unfolded or misfolded proteins in the ER, leading to ER-stress. Additionally, biotic and abiotic stress such as pathogen infection, high salt, and heat, can cause ER-stress (Che et al., 2010; Deng et al., 2011; Gao et al., 2008; Liu et al., 2007b; Liu et al., 2011a; Moreno et al., 2012). ER-stress can be induced by chemicals interfering with protein folding in the ER, such as Tunicamycin (TM) or Dithiothreitol (DTT). Unfolded ER proteins are degraded by ER-associated protein degradation (ERAD), a process involving re-localization of proteins into the cytoplasm where they are degraded in a 26S-proteasome-dependent manner (reviewed in Deng et al., 2013a). Specialized proteins in the ER sense unfolded proteins and activate the unfolded protein response (UPR) if too many unfolded proteins accumulate. It is therefore important that the UPR is suppressed in non-stress conditions to prevent spurious degradation of proteins. The UPR in *Arabidopsis* has two “arms”, one involving IRE1 (Inositol Requiring Enzyme 1), an ER transmembrane ribonuclease kinase which senses unfolded proteins in the ER lumen (Gardner

and Walter, 2011), and the other involving the ER transmembrane transcription factor (TF) bZIP28 (Liu et al., 2007a). The simultaneous loss of IRE1 and bZIP28 was shown to be lethal, emphasizing the importance of functional UPR during development (Deng et al., 2013b). During ER-stress IRE1 unconventionally splices *bZIP60* mRNA in the cytoplasm (Deng et al., 2011; Nagashima et al., 2011). *bZIP60* encodes a TF with a transmembrane domain (TMD) (Iwata and Koizumi, 2005). Splicing of *bZIP60* mRNA leads to a frameshift resulting in loss of the TMD and exposure of an NLS; the resulting bZIP60 protein is soluble and can enter the nucleus (Deng et al., 2011; Iwata et al., 2008; Nagashima et al., 2011; Zhang et al., 2015). In the nucleus, bZIP60 activates downstream UPR genes, such as the TF *NAC103* (Sun et al., 2013b). Under normal growth conditions, bZIP28 is bound by BIP chaperones and retained at the ER (Srivastava et al., 2013). Under ER-stress conditions, unfolded proteins compete BIP proteins away from bZIP28, and thereby enable bZIP28 to relocate from the ER to the Golgi where it is proteolytically processed so its cytoplasmic bZIP-containing domain can enter the nucleus (Liu et al., 2007a; Srivastava et al., 2013). Liu et al. (2007a) also reported that bZIP28 expression did not change in response to TM treatment, likely due to its regulation on the protein level. Although bZIP60 and bZIP28 were shown to heterodimerize with each other (Liu and Howell, 2010) and are functionally redundant in UPR (Sun et al., 2013a), they also bind target genes independently or with different affinity (Liu and Howell, 2010; Sun et al., 2013b).

The role of chromatin modifications in plant ER-stress responses is only emerging. A study by Song et al. (2015) revealed that ER-stress induced the deposition of H3K4me3, a mark associated with active gene expression, at the PcG target genes *SEC31A* and *BIP3*. This deposition was shown to be mediated by the COMPASS-like complex which is targeted to these genes by bZIP60 and bZIP28 (Song et al., 2015). In the same study it was also shown that the ER-stress-responsive genes *NSF*, *ERDJ3A*, *SARA1A* and *TIN1* did not acquire H3K4me3 during ER-stress, revealing that not all ER-stress-responsive genes are targeted by H3K4me3 for induction. They also showed that the bZIP60 induced TF NAC103 interacts with COMPASS-like complex members, indicating that NAC103 target genes are also regulated by histone methylation.

Early during ER-stress, UPR inhibits transcription and translation, activates genes which help the cell to deal with an excess of unfolded or misfolded proteins, such as the chaperones BIP3 (Noh et al., 2003) and ERDJ3A (Yamamoto et al., 2008), and induces genes which prevent programmed cell death (PCD), such as *BI1* (*BAX INHIBITOR 1*) (Watanabe and Lam, 2008). If ER-stress is prolonged and/or exceeds the protein folding capacity of the ER, PCD will be

induced (Watanabe and Lam, 2008). One inducer of cell death in *Arabidopsis* is the ER membrane associated TF NAC089, which relocates from the ER to the nucleus under ER-stress conditions to induce expression of downstream PCD regulators (Yang et al., 2014). *NAC089* is induced by both arms of the UPR, bZIP60 and bZIP28 (Yang et al., 2014), revealing that UPR induces expression of both pro-survival and pro-cell death genes.

Until now no negative regulator of ER-stress responses has been identified in *Arabidopsis*.

BLISTER (BLI) is a plant specific protein, which interacts with POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) (Schatlowski et al., 2010). PRC2 is a histone-modifying complex, which represses its target genes by trimethylation of H3K27 (H3K27me3) (reviewed in Derkacheva and Hennig, 2014). In our previous study (Kleinmanns et al., 2016 – Manuscript I), we analyzed the transcriptional profile of *bli-1* mutants: we showed that BLI is required for silencing of a subset of PRC2 target genes and that genes responding to abiotic stress responses were up-regulated in *bli-1* mutants. We also observed an enrichment of GO-terms that indicate ER-stress in *bli-1*. Here we show that a significant number of ER-stress-responsive genes is up-regulated in *bli-1*. Moreover, we analyzed how *bli* mutants respond to ER-stress and identified BLI as a negative regulator of the ER-stress response in *Arabidopsis*. We furthermore show that BLI localizes to Golgi vesicles, but not to the ER, in *Arabidopsis*. The sub-cellular localization of BLI appears to be only partially dependent on its NLS (nuclear localization signal) and NES (nuclear export signal) domains but is also regulated by its C-terminus.

Results

bli **mutants are hypersensitive to ER-stress**

We previously showed that in *bli-1* mutants GO-terms with relation to ER-stress response/ UPR (Unfolded Protein Response) are enriched among up-regulated genes (Kleinmanns et al., 2016 – Manuscript I). Here, we further dissected the role of BLI in regulation of ER-stress-responsive genes. By re-analyzing the genes mis-regulated in *bli-1* mutants, we revealed that a significant number of genes up-regulated in response to ER-stress was also up-regulated in *bli-1*, whereas no ER-stress-responsive genes were down-regulated (Table 1, full list in Supplemental Table 4). This suggests that BLI represses ER-stress-responsive genes under normal growth conditions. Interestingly, a significant number of ER-stress-responsive H3K27me3 target genes, a mark set by PRC2, was up-regulated in *bli-1*, indicating that BLI normally represses those genes together with PRC2.

Table 1: ER-responsive genes are highly up-regulated in *bli-1*.
A significant number of ER-stress-responsive genes was up-regulated in *bli-1*, whereas no ER-stress-responsive genes were down-regulated. A significant number of ER-stress-responsive genes in *bli-1* was also targeted by H3K27me3. Nagashima et al. (2011) induced ER-stress by Tunicamycin (TM) treatment. Statistical significance was analysed using Chi-square test with Yates correction; a p-value equal to or below 0.05 was considered as statistically significant.

	ER-stress-responsive genes	total no. genes	%	Chi square test (p-value)
genome wide (Nagashima et al., 2011)	152	24000	0.63	
***bli-1* up**	45	292	15.41	<0.0001
***bli-1* down**	0	244	0.00	-
***bli-1* H3K27me3 target genes**	7	208	3.37	<0.0001

Mis-regulation of ER-stress-responsive genes in *bli* mutants suggested that BLI is involved in the regulation of ER stress. To test this, we subjected *bli* mutants to prolonged ER-stress. To induce ER-stress, we used the drug Tunicamycin (TM). TM interferes with N-glycosylation in the ER leading to improper folding of proteins and therefore ER-stress/UPR.

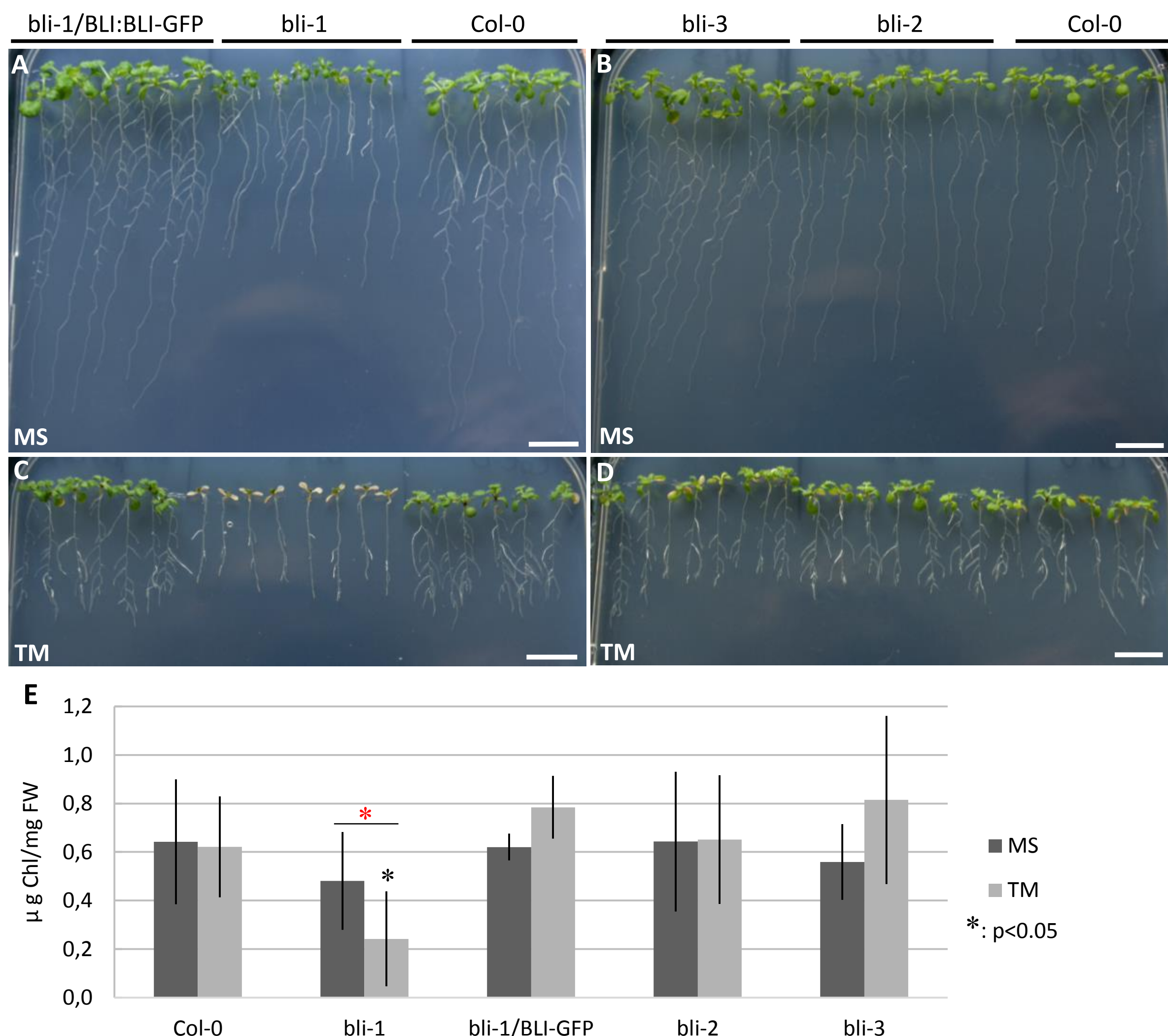

Figure 1: Response of *bli* mutants to ER-stress induced by Tunicamycin (TM).
A-D) Seedlings were grown vertically for 6 days on MS medium and then transferred to either MS or MS+0.3 µg/ml TM. After additional 6 days, *bli-1* seedlings grown on TM became strongly chlorotic, which was not the case for *bli-2* or *bli-3*, the complemented *bli-1*/BLI:BLI-GFP line, and Col-0 wild type. The TM concentration of 0.3µg/ml stopped root growth in all genotypes upon transfer. E) Only *bli-1* showed a significant reduction of total chlorophyll content after ER-stress treatment (TM) compared to i) non-stressed condition (MS; *) and ii) the wild type (*). Results of three independent experiments with

each two biological and three technical replicates are shown, ±SD of independent experiments. Statistical significance was analyzed using Student´s t-test, a p-value below 0.05 was considered as statistically significant.

We observed that neither the wild type nor the tested *bli* mutants or the complemented lines showed root growth after transfer to TM, whereas the control plants showed normal root growth on MS medium (Figure 1 A-D, Supplemental Figure 1). Notably, the strong *bli-1* and *bli-11* mutants are not only affected in general growth but also exhibit a short-root phenotype. *bli-1* and *bli-11* seedlings grown on TM became chlorotic and showed a reduced chlorophyll content (Figure 1 C, E, Supplemental Figure 1Supplemental Figure). This was not the case for the wild type, the weak *bli* mutants *bli-2* and *bli-3,* or the complemented *bli-1*/*BLI:BLI-GFP* line (Figure 1 C-E).

ER-stress-responsive genes are strongly up-regulated in non-stressed and ER-stressed *bli-1* mutants

To understand how loss of *BLI* affects expression of ER-stress-responsive gens upon ER-stress, we analyzed transcript levels of several highly up-regulated genes in *bli-1*. The tested genes comprise key transducers of ER-stress (*bZIP60*, *bZIP28*), an ER-stress induced ER chaperone (*BIP3*) and positive (*NAC103*) as well negative regulators (*NAC089*) of cell survival. We used the samples from our ER-stress experiment to examine the expression of those genes in non-stressed (MS) vs. ER-stressed (TM) seedlings. *bZIP60* was highly up-regulated in *bli-1* under non-stressed conditions, and its expression further increased under ER-stress conditions (Figure 2 A). Additionally, the weak *bli-2* mutant showed significantly higher *bZIP60* expression under non-stressed conditions compared to the wild type. *bZIP28* was not mis-regulated in *bli-1* in non-stress and ER-stress conditions (Figure 2 B). *BIP3* (*LUMINAL BINDING PROTEIN 3*) is not expressed under normal growth conditions, but its expression is induced by ER-stress (Iwata and Koizumi, 2005; Koizumi, 1996; Liu and Howell, 2010; Liu et al., 2007a; Martinez and Chrispeels, 2003; Nagashima et al., 2011). *BIP3* was expressed in non-stressed *bli-1,* and its expression increased about 5-fold upon ER-stress (Figure 2 C). *NAC103* expression is induced by bZIP60 upon ER-stress and in turn induces expression of down-stream UPR genes (Sun et al., 2013b). In non-stressed *bli-1, NAC103* was strongly up-regulated and its expression increased during ER-stress (Figure 2 D). *NAC089* encodes an ER membrane-associated TF that promotes ER-stress-induced programed cell death (PCD) (Yang et al., 2014). *NAC089* expression is induced by both branches of UPR, bZIP60 and bZIP28 (Yang et al., 2014), and its expression was significantly up-regulated in non-stressed and ER-stressed *bli-1* (Figure 2E).

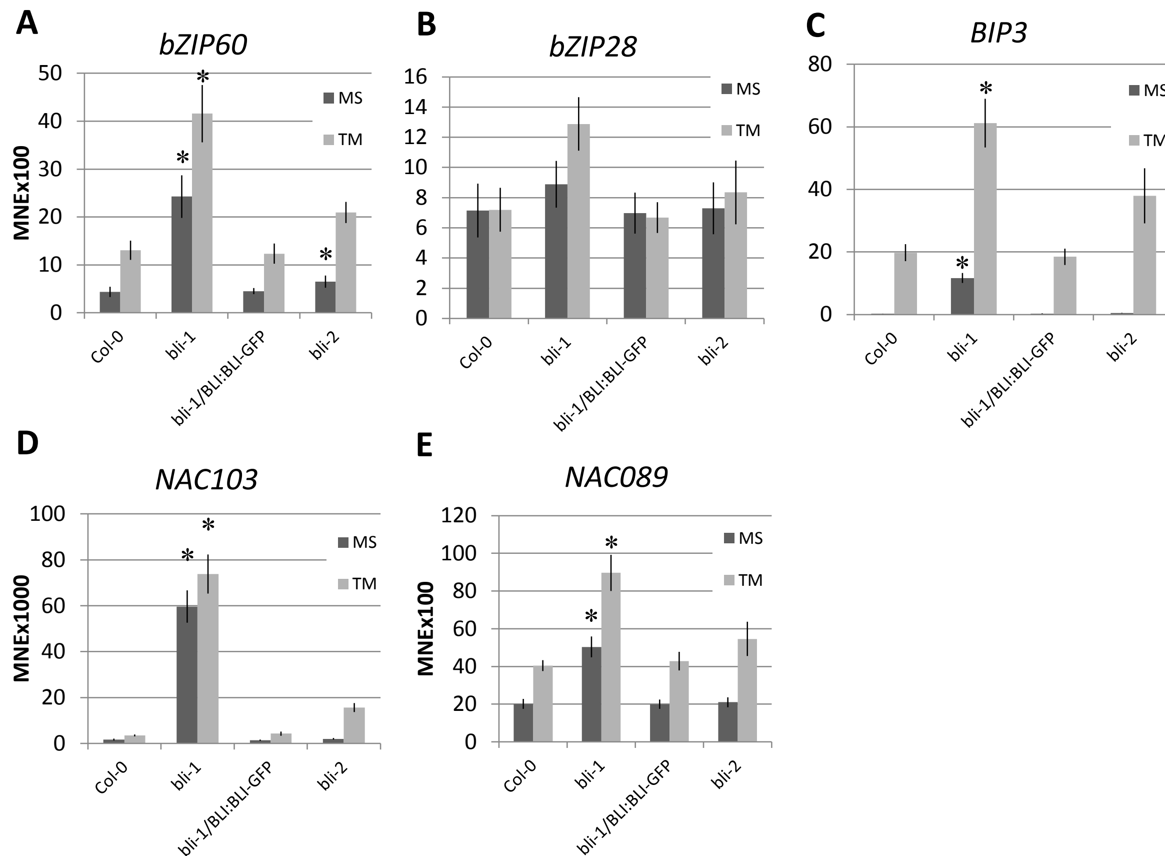

Figure 2: Expression of ER-stress-responsive genes in *bli* mutants with and without induction of ER-stress by Tunicamycin (TM).
ER-stress-responsive genes were significantly higher expressed in *bli-1* in the absence (MS) and during ER-stress (TM) compared to the wild type (Col-0), the complemented line *bli-1/BLI:BLI-GFP,* and the weak *bli-2* mutant. Only *bZIP28* expression was unchanged in all genotypes. Results from three independent experiments with two biological and three technical replicates each are shown; error bars show ±SE of three independent experiments. Statistical significance was analyzed using Student´s t-test, a p-value below 0.05 was considered as statistically significant.

To analyze the spatial expression pattern of an ER-stress-responsive gene, we studied *BIP3:GUS* (Maruyama et al., 2010) expression in *bli-11* seedlings (Figure 3). We expressed *BIP3:GUS* in *bli-11* because the *bli-1* mutant shows ectopic expression of the *LAT52:GUS* construct present on the SAIL T-DNA (Schatlowski et al., 2010). We observed a strong expression of *BIP3:GUS* in all tissues of *bli-11* 5 day old seedlings, whereas in 12-day-old seedlings the expression decreased in true leaves, showing a spotty *BIP3:GUS* expression pattern (Figure 3).

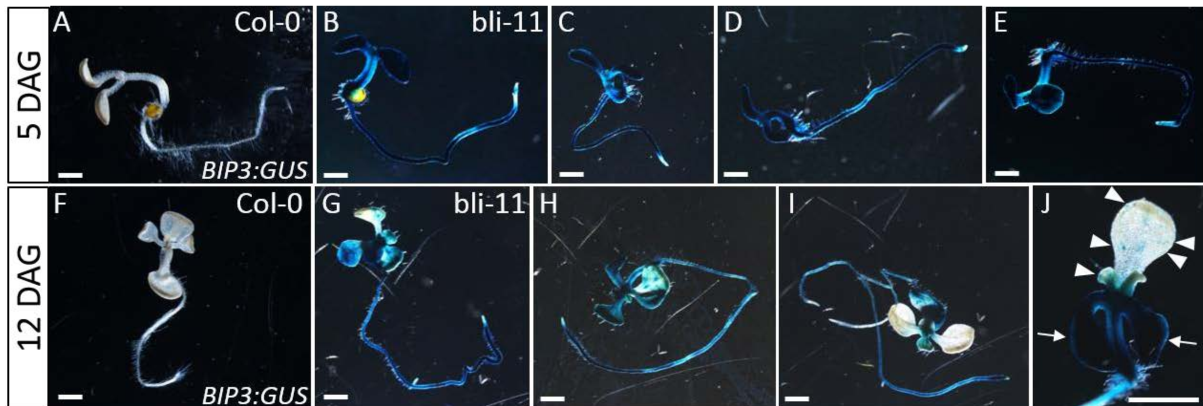

Figure 3: Expression of *BIP3:GUS* in *bli-11* 5 and 12 day old seedlings.
A-E) Expression of the ER chaperone *BIP3:GUS* in Col-0 (A) and *bli-11* (B-E) 5 day old seedlings. In contrast to the wt, *bli-11* shows strong expression of the *BIP3* reporter in all organs, which is in agreement with strong up-regulation of *BIP3* in *bli-1* plants. F-J) Expression of *BIP3:GUS* in Col-0 (F) and *bli-11* (G-J) 12 day old seedlings. The strong up-regulation of *BIP3:GUS* in *bli-11* is decreasing in differentiated tissue of 12 day old seedlings (G-I). Cotyledons (arrows in J) showed a strong expression of *BIP3:GUS*, whereas the GUS signal was weak or absent in true leaves (arrowheads in J). Scale bars are 500 µm.

In summary, *bli-1* plants which already suffer from ER-stress were highly susceptible to treatment with the ER-stress inducer Tunicamycin compared to all tested genotypes. ER-stress-responsive genes which are already up-regulated in *bli-1* became even higher expressed under ER-stress conditions. The weak *bli-2* mutant showed ectopic expression of *bZIP60* under non-stress conditions, revealing that even mildly reduced *BLI* expression leads to induction of ER-stress responses. These results strongly indicate that *BLI* is important for the negative regulation of ER-stress responses in *Arabidopsis* during normal growth and under ER-stress conditions.

Several ER-stress-responsive genes show increased levels of H3K4me3 in non-stressed *bli-1*

As BLI is interacting with the PcG proteins, we aimed to understand whether up-regulation of ER-stress-responsive genes in *bli-1* is associated with changes in specific histone modifications. Previously, we showed that up-regulation of the ER-stress-responsive PcG target genes *SEC31A* (*SECRETORY 31A*) and *BIP3* was not due to reduced levels of repressive H3K27me3 at these loci, whereas at least *SEC31A* acquired H3K4me3 (Kleinmanns et al., 2016 – Manuscript I). Here, our analysis revealed that *SEC31A* and *NAC103*, but not *BIP3*, acquired H3Kme3 in non-stressed *bli-1* mutants. Although the ER-stress-responsive genes *NSF*, *ERDJ3A*, *SARA1A,* and *TIN1* were up-regulated in *bli-1*, they did not acquire H3K4me3. The same was observed in ER-stressed wild type seedlings in the study by Song et al. (2015). Our ChIP analysis showed that most of the tested ER-stress-responsive genes behave similarly in *bli-1* as in ER-stressed wild type plants, indicating that BLI negatively regulates H3K4me3-associated changes in the ER-stress responses/UPR.

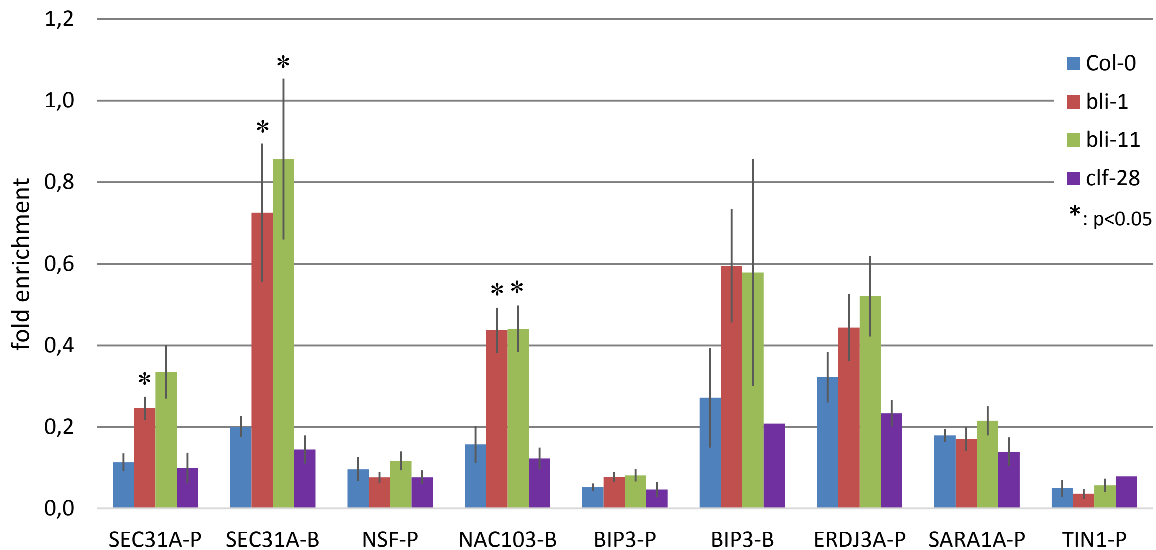

Figure 4: H3K4me3 levels at ER-stress-responsive genes in non-stressed wild type, *bli-1, bli-11*, and *clf-28*. Chromatin immunoprecipitation (ChIP) experiments revealed an enrichment of the activating mark H3K4me3 at ER-stress-responsive genes *SEC31A* and *NAC103*. The other tested ER-stress-responsive genes did not show enrichment of H3K4me3 but an increased expression in non-stressed *bli-1* (Supplemental Table 4). Oligonucleotides designed by Song et al. (2015), except for *NAC103*, were used in this study. Oligonucleotides 'P' were binding in the promoter region of a gene and 'B' in the gene body according to Song et al. (2015). Chromatin was precipitated using antibodies directed against H3K4me3 and was amplified by quantitative PCR. H3K4me3 levels at each locus were normalized to the *ACT7* locus. ChIP experiments were performed twice with 2 biological replicates each, and showed similar results. Quantitative PCR was performed with technical triplicates per sample. Error bars indicate ±SE of 2 independent ChIP experiments. Statistical significance was analyzed using Student´s t-test; a p-value below 0.05 was considered as statistically significant.

BLI does not localize to the ER but to nuclei and the Golgi in *Arabidopsis*

Key transducers of the UPR are localized at the ER. IRE1 is an ER transmembrane ribonuclease kinase which senses unfolded proteins in the ER lumen and then unconventionally splices *bZIP60* mRNA in the cytoplasm (Deng et al., 2011; Nagashima et al., 2011). Processed bZIP60 then enters the nucleus to activate down-stream UPR genes. bZIP28 is an ER-membrane-associated TF which moves to the Golgi upon ER-stress where it is cleaved and its cytoplasmic domain moves to the nucleus. As BLI negatively regulates ER-stress-responsive genes, it might act on the sites of ER stress signal transduction, i.e. the ER, Golgi, and/or nucleus. We previously showed that BLI-GFP fusion proteins localize to the nucleus and cytoplasmic 'speckles' in transient expression studies in *N. benthamiana* leaf epidermis cells (Schatlowski et al., 2010). Another study showed that C-terminal-truncated, 35S promoter driven BLI-GFP (KOS1-GFP) localized to nuclei and cytoplasmic speckles in *Arabidopsis* root cells (Purdy et al., 2010). Here we analyzed the expression pattern of full-length genomic *BLI* fused to *GFP* (*BLI:BLI-GFP*) in *Arabidopsis* root cells. In our previous study, we showed that the strong *bli-1* and *bli-11* mutants could be complemented when transformed with *BLI:BLI-GFP* (Kleinmanns et al., 2016 – Manuscript I) (see also Figure 1 A). Co-expression with the nuclear marker H2B-RFP (De Rybel et al., 2010) revealed that BLI-GFP localized to nuclei in cells of the root elongation zone but not in root tip cells (Figure 5 A-F). To test if BLI also localizes to the ER,

as indicated by its role in the ER-stress response, we analyzed stable double transgenic lines expressing BLI-GFP and the ER-marker WAK2-RFP (Nelson et al., 2007). The co-expression analysis clearly revealed that BLI-GFP does not localize to the ER (Figure 5 G-L).

However, we observed that BLI-GFP partially colocalized with the Golgi marker SYP32-RFP (WAVE22 (Geldner et al., 2009)) in *Arabidopsis* root cells (Figure 6 A-F). BLI-GFP did not colocalize with a Trans-Golgi/early endosome marker (VTI12-RFP/WAVE13 (Geldner et al., 2009)) (Figure 6 G-L). While BLI-GFP and VTI12-RFP signals were usually adjacent to each other, time-series imaging revealed that the two markers were not overlapping (Supplemental Movie 1). This indicates that the presence of BLI-GFP at the Golgi is likely not a signal for future secretion or degradation. Importantly, BLI-GFP does not contain any transmembrane domains (Figure 7 G), Golgi localization sequences or predicted myristoylation sites, therefore it is unclear whether BLI-GFP is present inside of or externally anchored to Golgi vesicles. In co-expression studies in *N. benthamiana* leaf epidermis cells, we could furthermore show that BLI-GFP partially colocalized with processing bodies (p-bodies) marked with RFP-DCP1 (Moreno et al., 2013) (Supplemental Figure 2). P-bodies contain de-capping enzymes, a 5′-to-3′ exoribonuclease, de-adenylases, RNAi machinery component AGO1, tandem zink finger proteins, and components of the nonsense-mediated mRNA decay pathway (reviewed in Maldonado-Bonilla, 2014). Whether BLI-GFP co-localizes with p-bodies in *Arabidopsis* needs to be analyzed in the future.

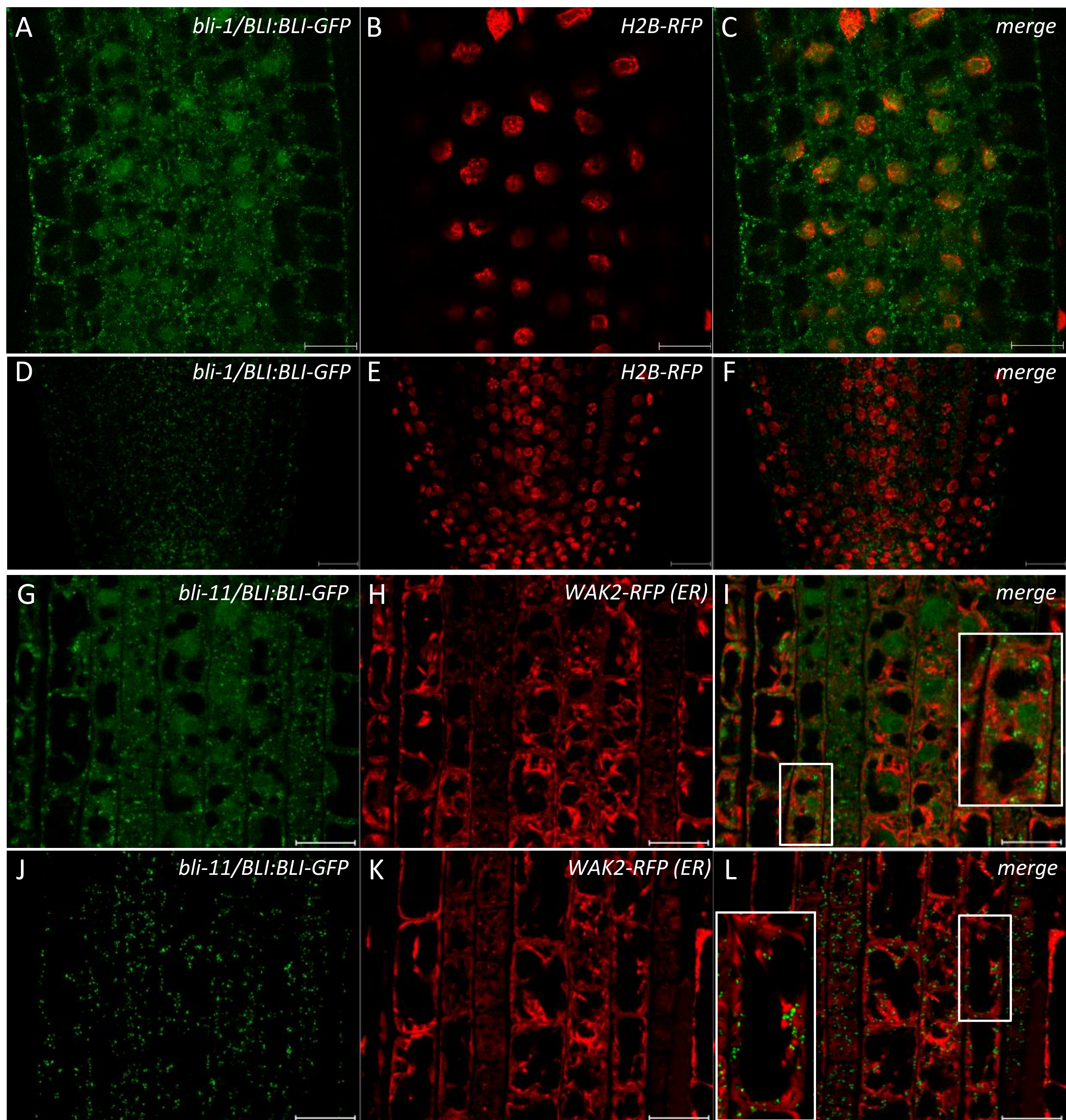

Figure 5: Laser scanning confocal imaging of *Arabidopsis* root cells expressing *BLI:BLI-GFP* and marker for nuclei (H2B-RFP, (De Rybel et al., 2010)) and the ER (WAK2-RFP (Nelson et al., 2007)).
A-F) BLI-GFP was present only in nuclei of the root elongation zone (A-C), but not root tip cells (D-F). G-L) BLI-GFP did not co-localize with the ER marker WAK2-RFP (close ups in I and L). Scale bars are 20 µm

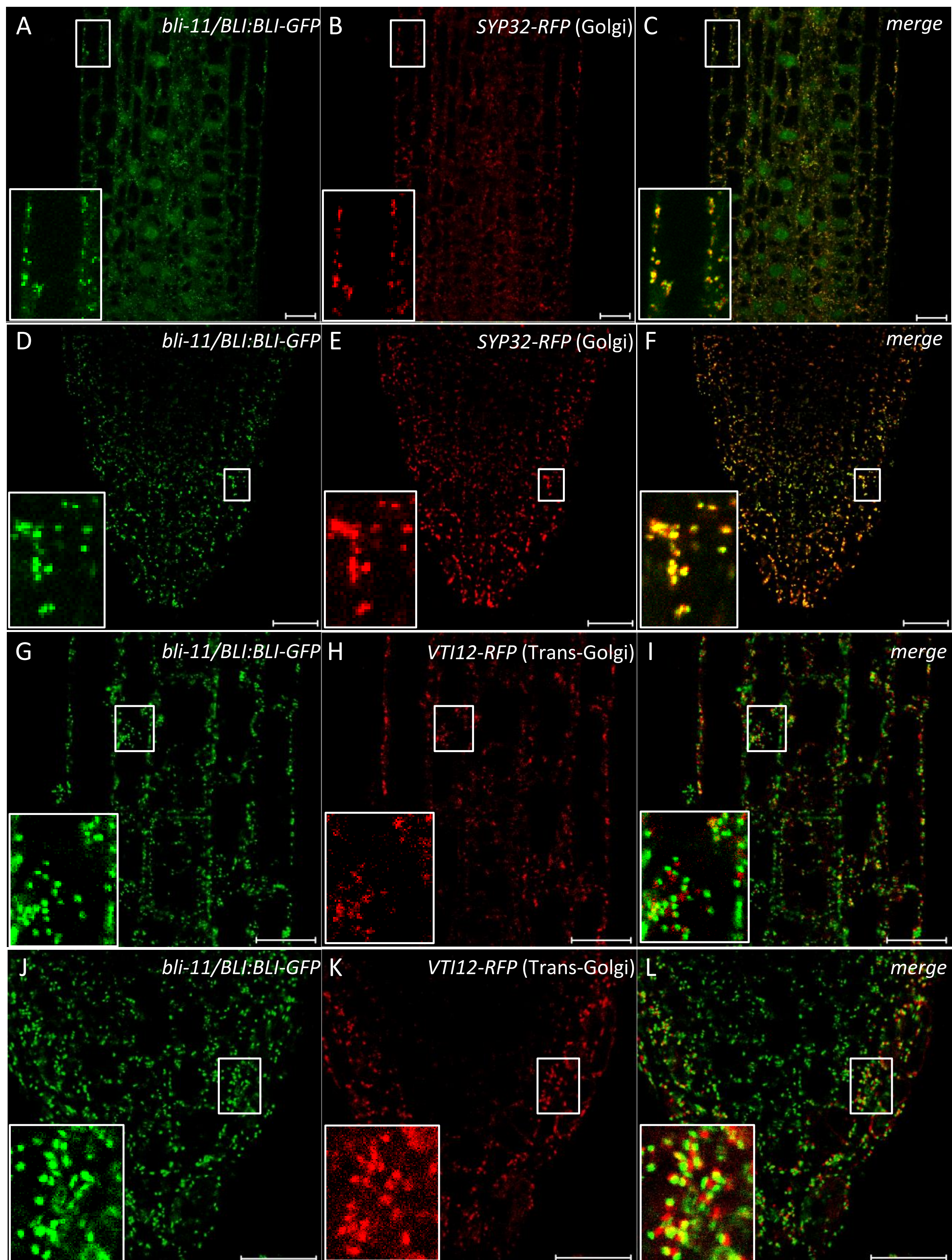

Figure 6: Laser scanning confocal imaging of *Arabidopsis* root cells expressing *BLI:BLI-GFP* and marker for the Golgi (SYP32-RFP/WAVE22 (Geldner et al., 2009)) and the Trans-Golgi/early endosome (VTI12-RFP/WAVE13 (Geldner et al., 2009)).
A-F) BLI-GFP colocalized with Golgi vesicles in all cell types, although the overlap was not complete (see close ups). G-L) BLI-GFP did not co-localize with the Trans-Golgi network (see close ups), time-series imaging (Supplemental Movie 1) revealed that BLI-GFP and VTI12-RFP signal were adjacent but not overlapping. Scale bars are 20 µm.

Mutation of BLI domains does not stably alter its localization in *Arabidopsis*

We reasoned that localization of BLI to different organelles and its function in the ER-stress response and PcG mediated gene regulation is controlled by different domains of the BLI protein. Therefore, interfering with the localization of BLI in a specific compartment might unravel the contribution of the different domains to BLIs role in developmental control or in stress responses. We therefore introduced point mutations in BLI´s predicted NLS and NES (Figure 8 G) and added an additional N-terminal NLS to BLI in order to force its localization to the nucleus. We first screened mutated *i35S:cBLI-GFP* constructs in *N. benthamiana* to test if the constructs are functional *in planta* (Supplemental Figure 3). Then we expressed mutated and NLS-tagged *BLI:cBLI-GFP* constructs in *bli-1* and *bli-11* mutants to test for the complementation ability and localization of mutated BLI-GFP (Figure 7 and Figure 8).

The non-mutated *BLI:cBLI-GFP* construct was able to complement the strong *bli-1* and *bli-11* phenotype (Figure 7 B). *BLI:cBLI-GFP* showed the same expression pattern as full length *BLI:BLI-GFP*; BLI-GFP localized to cytoplasmic speckles and to nuclei of the root elongation zone (Figure 8 A-C).

Mutation of the BLI NLS led to an abolished nuclear localization in *N. benthamiana,* but localization in cytoplasmic speckles was not affected (Supplemental Figure 3 D-F). In *Arabidopsis,* mutation of the NLS unexpectedly led to an abolished nuclear localization only in some lines but not all (Figure 8 D-F). Importantly, the construct carrying a mutation in the BLI NLS could fully complement the strong *bli-1* and *bli-11* phenotypes in all lines (Figure 7 C).

Because BLI-GFP was only found in nuclei of cells of the root elongation zone but not in root tip cells or cells above the elongation zone, we wanted to understand which effect a constitutive nuclear localization of BLI would have. To test this, we added the strong simian virus 40 (SV40) large T-antigen NLS (PKKKRKV) (Kalderon et al., 1984), which is functional in plants (van der Krol and Chua, 1991), to the BLI N-terminus. In most lines the additional NLS led to nuclear, but not nucleolar, localization of BLI-GFP in all root cell types. Interestingly, the SV40 NLS could not abolish BLI-GFP localization in cytoplasmic speckles/Golgi (Figure 8 G-J). Strikingly, some lines did not show nuclear localization at all, but always cytoplasmic speckle/Golgi localization of BLI-GFP. Importantly, the SV40-NLS-tag construct could complement the strong *bli-1* and *bli-11* mutant phenotype without causing additional phenotypes (Figure 7 D, E).

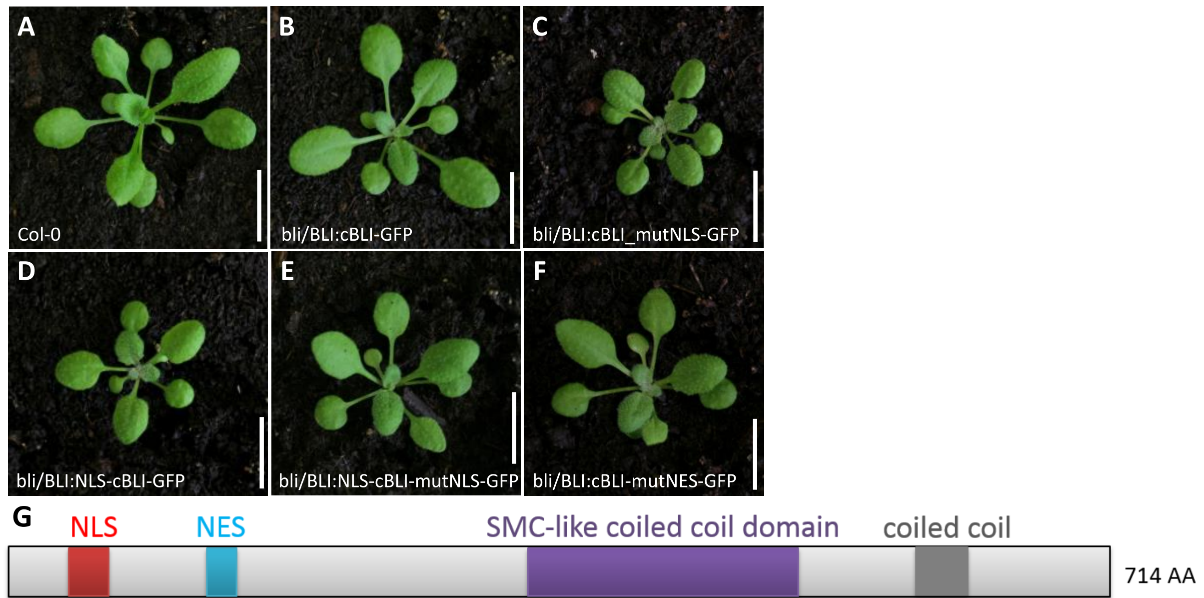

Figure 7: Expression of mutated *BLI:cBLI-GFP* in *bli* mutants.
A-F) Phenotypes of the wild type (A), and *bli* mutants expressing non-mutated BLI-GFP (B) and mutated BLI-GFP versions (C-F). Expression of non-mutated as well as mutated BLI-GFP in *bli* could complement the *bli* mutant phenotype (B-F). G) Schematic representation of the BLI protein domains. NLS=Nuclear Localization Signal; NES=Nuclear Export Signal; SMC-like coiled coil domain= Structural Maintenance of Chromosomes-like domain, important for interaction with CLF.

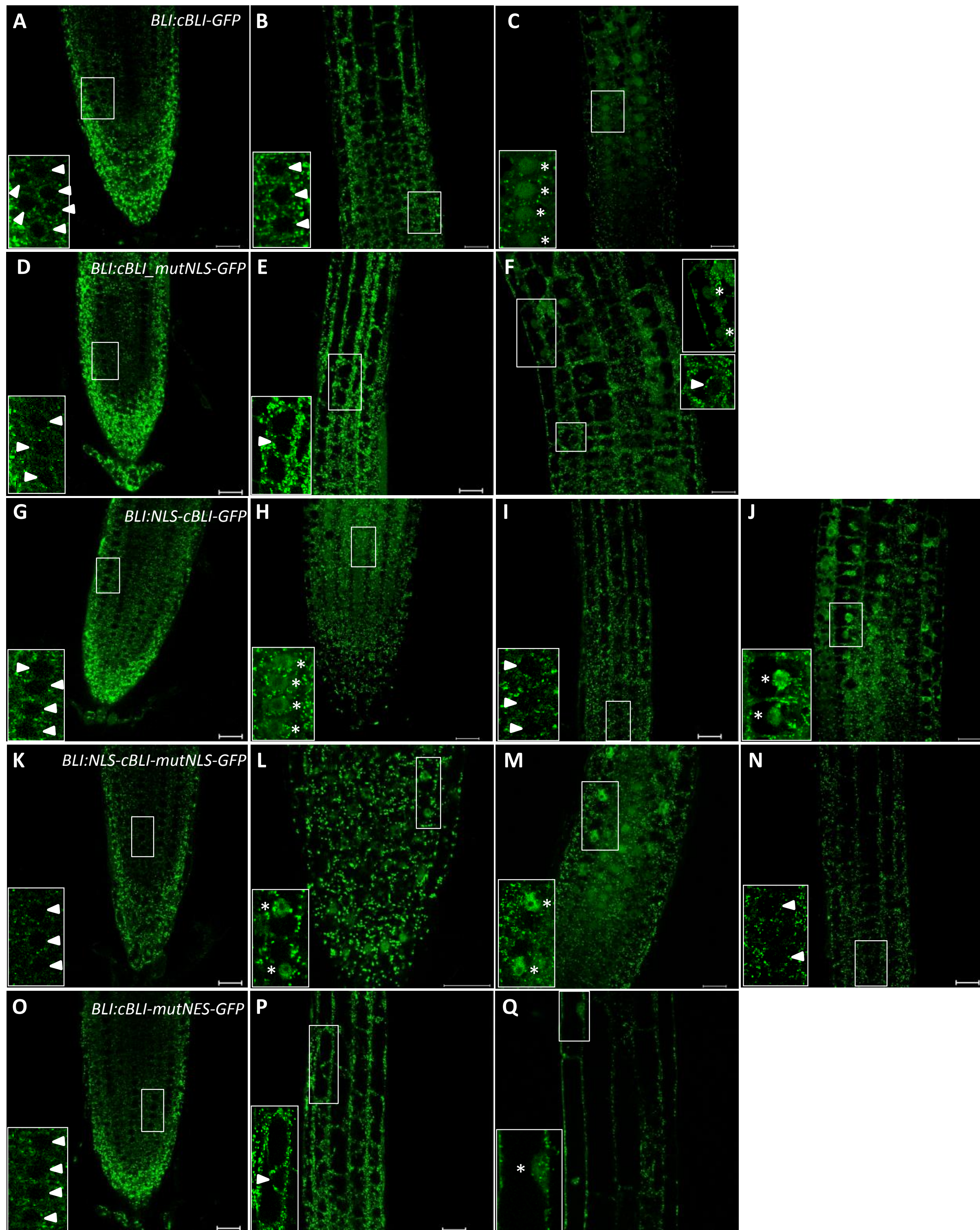

Figure 8: Expression of mutated *BLI:cBLI-GFP* in *Arabidopsis* root cells.
A-C) Laser scanning confocal pictures of non-mutated BLI-GFP. Arrowheads indicate a non-nuclear localization of BLI-GFP, asterisks indicate nuclear localized BLI-GFP. (D-F) Mutation of the NLS in cBLI (*BLI:cBLI_mutNLS-GFP*) could not completely abolish nuclear localization of cBLI-GFP. (G-J) Expression of BLI-GFP with an N-terminal NLS tag. Some lines showed nuclear localized cBLI-GFP in all cell types (H, J, L, M), but some only showed localization in cytoplasmic speckles (G, I, K, N). (O-Q) The mutation of cBLI NES (BLI:cBLI_mutNES-GFP) did not alter the subcellular localization compared to the non-mutated cBLI-GFP (A-C). Scale bars are 20 µm.

The BLI C-terminus is important for localization in cytoplasmic speckles

In our previous study, we expressed truncated BLI without a C-terminus (N/SMC-BLI-GFP) in *N. benthamiana* leaf epidermis cells (Schatlowski et al., 2010). N/SMC-BLI-GFP localized

strongly to nuclei and only weakly to the cytoplasm but not to cytoplasmic speckles (Schatlowski et al., 2010). To understand if the C-terminus of BLI carries the domain responsible for localization in cytoplasmic speckles, we expressed inducible *i35S:N/SMC-cBLI-GFP* in *bli-1*. N/SMC-BLI-GFP localized to nuclei and weakly to the cytoplasm of *Arabidopsis* root cells, but not to cytoplasmic speckles (Figure 9 D-F). This localization pattern clearly indicates that the BLI C-terminus contains a sequence necessary for localization at the Golgi. The C-terminus of BLI contains a coiled-coil domain (Figure 7 G), which could mediate the localization of BLI-GFP in cytoplasmic speckles, i.e. Golgi and p-bodies, possibly by protein-protein interaction.

BLIs SMC domain is responsible for interaction of BLI and the PcG protein CLF (CURLY LEAF) (Schatlowski et al., 2010). Expression of the BLI SMC domain in *Arabidopsis* root cells resulted in strong nuclear and weak cytoplasmic localized SMC-GFP (Figure 9 G-I). The SMC domain does not contain an NLS but localized to nuclei, indicating that this domain can contribute to nuclear presence of BLI. The observed nuclear localization of BLI containing a mutated NLS (Figure 8 D-F) could hence be mediated by the SMC domain.

Taken together, expression of mutated or truncated BLI-GFP in *Arabidopsis* revealed a different regulation of BLI in *Arabidopsis* and *N. benthamiana*. Furthermore, we observed that nuclear localization of BLI is probably dependent on more than one protein domain and is likely controlled via additional mechanisms.

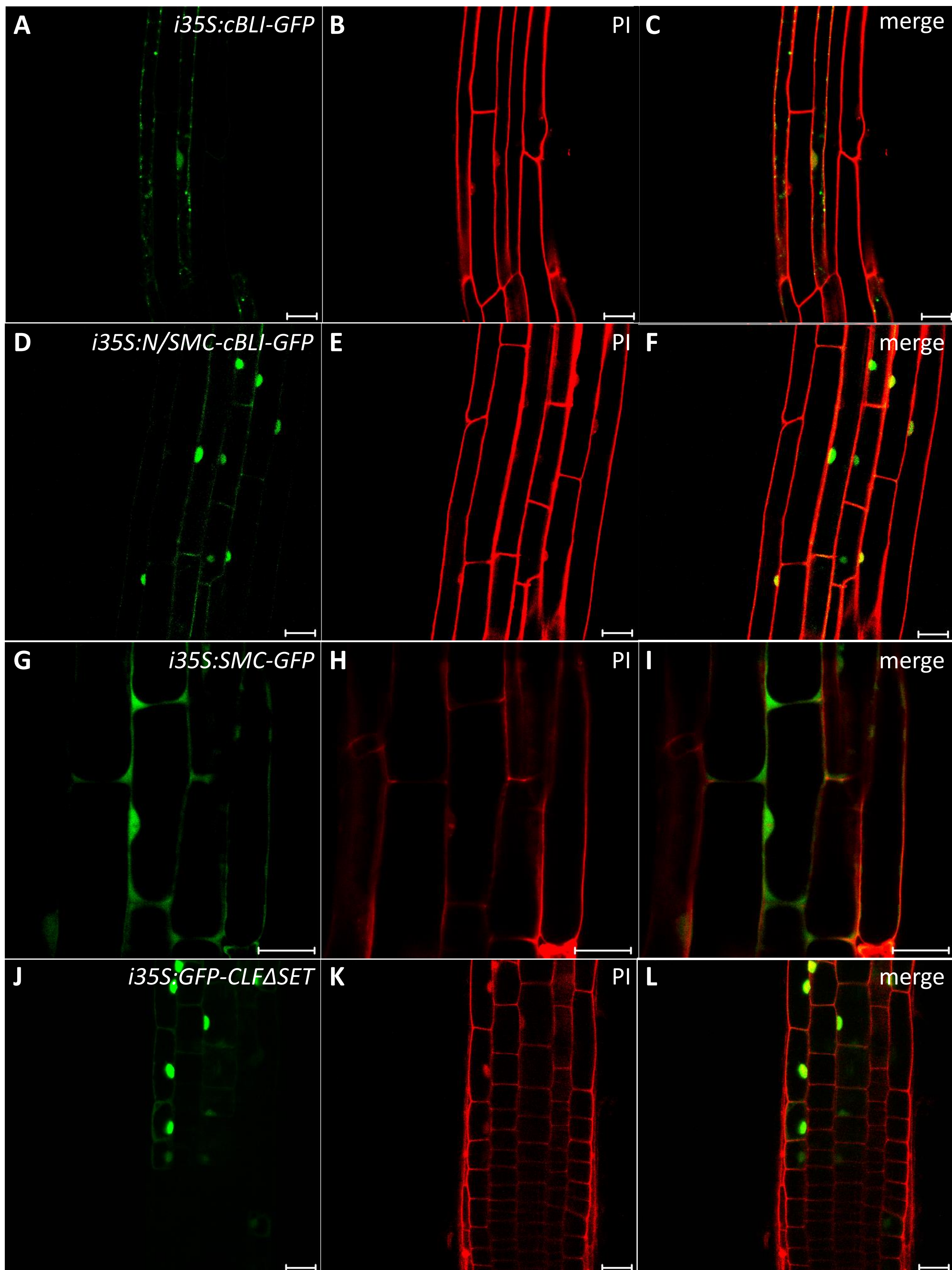

Figure 9: Expression of truncated *i35S:cBLI-GFP* in *Arabidopsis* root cells.
A-C) Expression of *i35S:cBLI-GFP* in *Arabidopsis* root cells revealed a localization of BLI-GFP in nuclei and cytoplasmic speckles. D-F) N/SMC-BLI-GFP localized to nuclei and weakly to the cytoplasm in *Arabidopsis* root cells. No localization in cytoplasmic speckles could be observed. G-I) The BLI SMC domain fused to GFP localized to nuclei and weakly to the cytoplasm. J-L) GFP-CLFΔSET, lacking the histone methyltransferase SET domain, localized to nuclei and also weakly to the cytoplasm of *Arabidopsis* root cells. Expression of constructs was induced by 10 µM ß-estradiol for 12 h. PI: propidium iodide Scale bars are 20 µm.

Discussion

ER-stress responses in plants can be caused by extensive gene expression during development, as well as by abiotic and biotic stress (Che et al., 2010; Deng et al., 2011; Gao et al., 2008; Liu et al., 2007b; Liu et al., 2011a; Moreno et al., 2012). When too many unfolded proteins accumulate in the ER, the unfolded protein response (UPR) is triggered. UPR not only activates genes which help the cell to deal with an excess of unfolded or misfolded proteins but also induces genes which prevent programmed cell death (PCD). However, excessive ER-stress will lead to PCD when the folding capacity of the ER is exceeded. In non-stress conditions, it is important that the UPR is suppressed to prevent spurious degradation of proteins. So far no suppressor of ER-stress or the UPR has been identified in plants.

We previously showed that BLI is involved in the regulation of responses to abiotic and biotic stress and that ER-stress-responsive genes were up-regulated in *bli-1* mutants (Kleinmanns et al., 2016 – Manuscript I). The latter indicated that BLI might be involved in the negative regulation of ER-stress responses. Therefore, we here dissected the role of BLI in ER-stress responses. In *bli-1* mutants, a high number of ER-stress-responsive genes was up-regulated under non-stress conditions (Table 1 and Supplemental Table 4).We therefore tested how *bli* mutants respond to ER-stress. We observed that strong, but not weak, *bli* mutants are highly susceptible to ER stress induced by the drug Tunicamycin (TM) (Figure 1, Supplemental Figure 1). To understand which functional category of ER-stress-responsive genes is activated in *bli*, we quantified the expression of key genes involved in UPR (*bZIP60*, *bZIP28*, *BIP3*) as well as genes involved in pro-survival (*NAC103*) or pro-cell death (*NAC089*) responses (Figure 2). Expression of UPR transducer bZIP60 but not bZIP28 is increased in response to TM treatment (Iwata et al., 2008; Liu et al., 2007a), revealing a different regulation of these TFs. In *bli-1,* expression of *bZIP60* was strongly increased in non-stress and ER-stress conditions, and *bli-2* showed up-regulation of bZIP60 under non-stress conditions. This indicates that even a mild reduction of *BLI* expression, as is the case in *bli-2* mutants, leads to induction of ER-stress responses. bZIP28 expression did not change significantly in *bli* mutants, which is likely due to its regulation on the protein level. To understand if the bZIP28 pathway is affected in *bli-1*, we analyzed the expression of one of the bZIP28 main target genes *BIP3* (Liu and Howell, 2010). *BIP3* was highly expressed in non-stressed and even higher in ER-stressed *bli-1* (Figure 2 and Figure 3). Although expression of *BIP3* can also be induced by bZIP60 (Iwata and Koizumi, 2005), we conclude that the high expression of *BIP3* in *bli-1* is due to activation of both UPR arms. Importantly, overexpression of *BIP3* could also retain bZIP28 in the ER

(Srivastava et al., 2013). Therefore, the analysis of bZIP28 localization in *bli-1* non-stressed and stressed plants will reveal how bZIP28 contributes to the upregulation of ER-stress-responsive genes in *bli-1*. bZIP60 activates expression of the pro-survival TF NAC103 (Sun et al., 2013b). *NAC103* expression was strongly increased in both non-stressed and ER-stressed *bli-1* seedlings. On the other hand, the pro-cell death TF NAC089 was also strongly expressed in non-stressed and ER-stressed *bli-1*, indicating that prolonged ER-stress treatment finally induced PCD in *bli-1*. This is consistent with the observation that *bli-1* plants did not survive prolonged ER-stress, and showed necrosis and loss of chlorophyll (Figure 1). Since the loss of BLI leads to ectopic and increased expression of ER-stress-responsive genes under normal growth and ER-stress conditions, we propose that BLI is a negative regulator of ER-stress responses/UPR during normal growth and under ER-stress conditions.

The role of chromatin modifications in ER-stress responses is only emerging. The H3K18Ac deacetylase SIRT7 for example represses transcription of ribosomal proteins in response to ER-stress, in order to prevent accumulation of unfolded proteins until ER homeostasis is reestablished (Shin et al., 2013). H4R3 methylation and H4 acetylation were shown to be induced by ER-stress at the *GRP78/BIP* promoter in human cell lines (Baumeister et al., 2005). The H3K4me3-binding protein SGF29 plays a central and dual role in the ER-stress response in animals. Prior to ER-stress, the protein coordinates H3K4me3 levels, thereby maintaining a 'poised' chromatin state on ER-stress target gene promoters (Schram et al., 2013). Following ER-stress induction, SGF29 is required for increased H3K14 acetylation on these genes, which then results in full transcriptional activation, thereby promoting cell survival (Schram et al., 2013). In contrast to animals, ER-stress-responsive genes in plants do not carry H3K4me3 prior to ER stress (Song et al., 2015). A recent study in *Arabidopsis* showed that TM treatment induced H3K4me3 at several ER-stress-responsive gene promoters and that loss of bZIP60 and bZIP28 function impaired the H3K4me3 occupancy at those genes (Song et al., 2015). Moreover, Song et al. (2015) showed that bZIP60 and bZIP28 directly interact with members of the COMPASS-like complex, which interacts with histone lysine methyltransferases to set H3K4me3. To understand if *bli-1* mutants are affected in this histone modification, we tested the same genes and regions as Song et al. (2015) for H3K4me3 occupancy. We could reveal that several genes in non-stressed *bli-1* acquired H3K4me3 (Figure 4). One of these genes is a PcG target gene, the other is not, indicating that BLI restricts H3K4me3 during non-stress conditions at PcG target and non-target genes. It is to mention, that genes regulated by H3K4me3 were not significantly enriched among mis-regulated genes in *bli-1* (Kleinmanns et al., 2016 – Manuscript I). Therefore, BLI is not generally involved in the counteraction of

H3K4me3 levels in *Arabidopsis*, but likely restricts deposition of this mark only at specific, stress-related H3K27me3 target and non-target genes (Figure 10). Importantly, increase of H3K4me3 at *SEC31A* and *NAC103* could also be indirectly regulated by BLI: loss of BLI could induce ER-stress, leading to increased levels of H3K4me3 at *SEC31A* and *NAC103* in *bli-1*. BLI would therefore not restrict binding of TrxG proteins to these genes under non-stress conditions, but would regulate transducers of ER-stress responses, which activate *SEC31A* and *NAC103* by recruiting TrxG proteins to these loci in response to ER-stress. However, our analysis revealed that most of the tested ER-stress-responsive genes behave similarly in non-stressed *bli-1* mutants as in ER-stressed wild type plants and hence further reveal that BLI is a negative regulator of ER-stress responses/UPR under normal growth conditions. Whether BLI directly associates with ER-stress-responsive genes or interacts with bZIP60, bZIP28, TrxG proteins, or the COMPASS-like complex to restrict H3K4me3 deposition at ER-stress-responsive genes under non-stress conditions currently remains unresolved.

In animals and yeast several pathways are described which alleviate ER-stress. In mice the H3K18Ac deacetylase SIRT7 functions at chromatin to suppress ER stress (Shin et al., 2013). SIRT7 is induced upon ER stress and is stabilized at the promoters of ribosomal proteins through its interaction with the transcription factor Myc to silence gene expression and to relieve ER stress (Shin et al., 2013). In contrast to SIRT7, BLI is not up-regulated in response to ER-stress (Iwata et al., 2008; Kamauchi et al., 2005; Nagashima et al., 2011). Therefore, BLI might act on the protein level to prevent ER-stress, like bZIP28. Another study in mice showed that CYP2J2-derived EETs (epoxyeicosatrienoic acids) act as important regulators of intracellular Ca^{2+} levels and ER homeostasis (Wang et al., 2014). *CYP2J2* expression significantly reduced ER stress and associated apoptosis, and attenuated the development of heart failure in mice (Wang et al., 2014). In yeast, a constitutive activation of the heat shock response (HSR) could increase ER stress resistance in both wild-type and UPR-deficient cells, mainly through facilitation of protein folding and secretion (Hou et al., 2014). These studies in animals and yeast describe proteins and processes which alleviate ER-stress after initiation but could not prevent it. BLI likely prevents ER-stress during development and under stress conditions, hence is acting upstream of the ER-stress response.

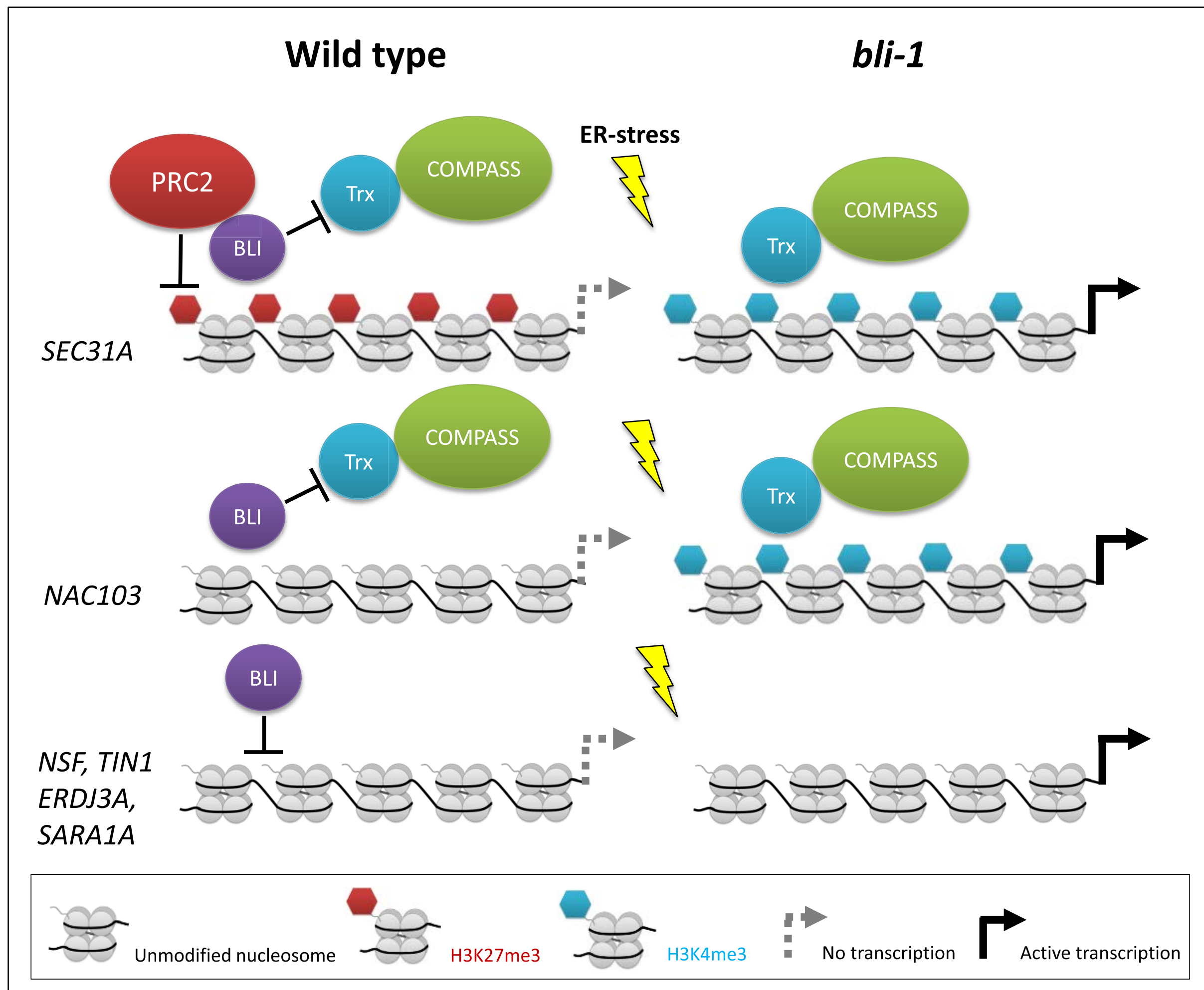

Figure 10: Regulation of ER-stress-responsive genes by BLI.
In the wild type (left), BLI represses the expression of the ER-stress-responsive genes *SEC31A*, *NAC103*, *NSF*, *TIN1*, *ERDJ3A*, and *SARA1A*. Repression of the H3K27me3 target gene *SEC31A* by BLI likely occurs in concert with PRC2. At *SEC31A* and *NAC103,* BLI likely prevents binding of TrxG proteins/COMPASS-like complex, hence restricting setting of the activating H3K4me3 mark at these loci. In *bli-1* mutants (right) ER-stress-responsive genes are activated; probably the loss of BLI causes ER-stress. The expression of several key UPR genes (Figure) showed an even stronger increase in expression in ER-stressed *bli-1* mutants compared to non-stress conditions. This indicates that BLI prevents ectopic expression of these genes during normal growth conditions and restricts maximum expression under ER-stress conditions.

bZIP60 and bZIP28 both encode TFs with a transmembrane domain. To enter the nucleus in response to ER-stress, bZIP60 mRNA needs to be spliced by IRE1 whereas bZIP28 is subject to proteolytical cleavage at the Golgi (Deng et al., 2011; Liu et al., 2007a; Nagashima et al., 2011). To understand how BLI is involved in ER-stress regulation, we also analyzed the subcellular localization of BLI-GFP fusion proteins in *Arabidopsis* (see Figure 11). BLI-GFP mostly localized to nuclei of the root elongation zone in *Arabidopsis* (Figure 5) but did not localize to the ER. BLI-GFP colocalized with a Golgi marker (Figure 6), although no Golgi retention or localization signals and no myristoylation sites are present in the BLI protein sequence. Importantly, we did not observe colocalization of BLI and a trans-Golgi/early endosome marker, revealing that presence of BLI at the Golgi neither leads to secretion nor degradation (Figure 6). The presence of BLI-GFP at the Golgi could indicate an interaction of

BLI with bZIP28 during ER-stress. Interaction studies will reveal whether BLI associates with Golgi proteins which could retain BLI at the Golgi during normal growth.

Nuclear import of a protein can be achieved by at least four mechanisms which are not mutually exclusive: 1) modification (e.g. phosphorylation) or change of conformation which will expose an NLS or mask an NES, 2) formation of heterodimers or heterocomplexes which allow import by a 'piggyback' mechanism, 3) cytoplasmic retention by protein-protein interactions and its release by protein modifications, and 4) cytoplasmic retention by membrane association and its release by proteolysis (reviewed in Meier and Somers, 2011). Moreover, proteins lacking a NLS were shown to be imported into the nucleus by direct interaction with the nuclear pore complex (Fagotto et al., 1998). The nuclear localization of bZIP60 is dependent on an NLS that is exposed after splicing by IRE1 (Zhang et al., 2015). The mechanism of bZIP28 nuclear import is still unclear although the bZIP domain was shown to function as a nuclear targeting signal (van der Krol and Chua, 1991). BLI contains several domains, an NLS, NES, SMC-like domain, and a coiled coil domain (Figure 7G). To understand which domains of BLI are responsible for its localization in nuclei and the Golgi, we mutated several BLI domains and introduced a strong N-terminal NLS tag. Moreover, we induced expression of truncated versions of BLI-GFP in *Arabidopsis*. The localization of the mutated BLI-GFP versions in *N. benthamiana* revealed their functionality *in planta*. Strikingly, the localization observed in *N. benthamiana* was different from the localization in *Arabidopsis* root cells. All tested mutated constructs could rescue the strong *bli-1* and *bli-11* mutant phenotype (Figure 7). Unexpectedly, mutation of the NLS was not sufficient to abolish nuclear localization of BLI-GFP in *Arabidopsis*, as was the case in *N. benthamiana* (Figure 8 and Figure 7). Expression of the SMC-GFP in *Arabidopsis* revealed that this domain itself confers nuclear localization (Figure 9). Therefore, both the NLS and SMC domain could be important for nuclear localization of BLI. The mutation of BLIs NES did neither abolish the cytoplasmic localization of BLI-GFP nor did it confer complete nuclear localization, indicating a regulation of BLI localization by additional mechanisms than by its NLS and NES. When we expressed BLI-GFP containing an NLS tag from the simian virus 40 (SV40) large T-antigen, we expected BLI-GFP to completely localize to nuclei of all cell types as described before (van der Krol and Chua, 1991). Instead, we observed that in some lines BLI-GFP was nuclear-localized in all cell types, whereas in other lines the SV40 NLS was not sufficient for nuclear localization of BLI (Figure 7). Importantly, all lines showed BLI-GFP localization in the cytoplasm, indicating that even a strong NLS cannot confer import of all BLI-GFP molecules present in a cell, suggesting that there potentially is a strong retention signal keeping BLI at the Golgi.

N/SMC-BLI-GFP lacking the BLI C-terminus (Schatlowski et al., 2010) localized to nuclei and weakly to the cytoplasm when expression was induced in *Arabidopsis* root cells (Figure 9). Localization in cytoplasmic speckles was no longer detectable. The BLI C-terminus contains a coiled coil domain which could confer Golgi localization of BLI via protein-protein interaction. We cannot exclude the possibility that the C-terminus of BLI, conferring localization in cytoplasmic compartments (Golgi), is cleaved off and free BLI (without GFP) then enters the nucleus in cells outside of the elongation zone. Deletion of this coiled coil domain will reveal if the Golgi localization of BLI is dependent on this domain and if this deletion can confer nuclear localization of BLI.

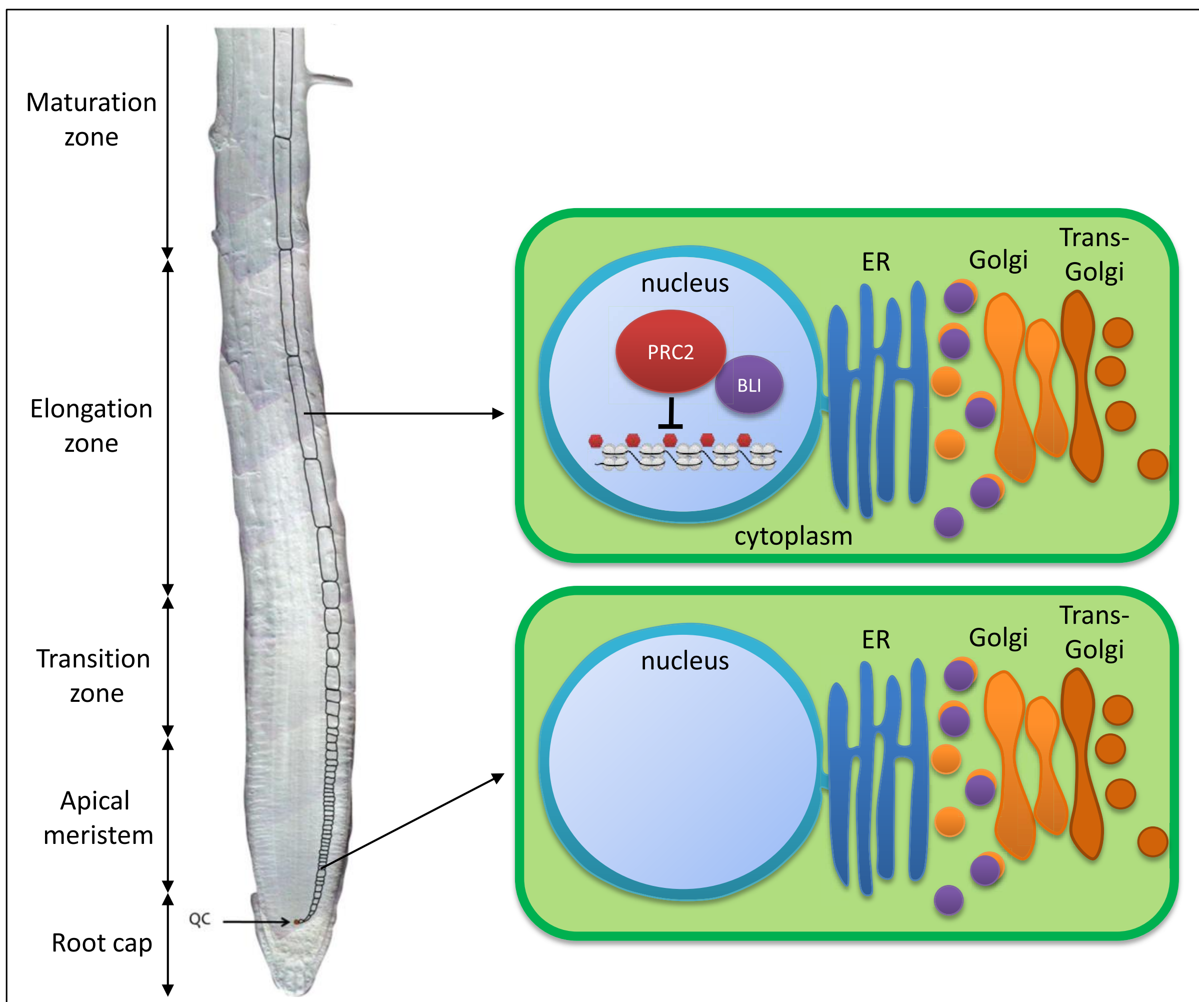

Figure 11: Overview of BLI localization in *Arabidopsis* root cells.
BLI-GFP localized to nuclei and the Golgi in cells of the root elongation zone. BLI-GFP did not colocalize with the ER or the Trans-Golgi network. In nuclei BLI likely interacts with PRC2 to regulate gene expression (upper cell), but probably also acts independent of PRC2. In cells of the root tip (Apical meristem and transition zone; lower cell) BLI-GFP localized to the Golgi but not to nuclei. We cannot exclude the possibility that the C-terminus of BLI, conferring localization in cytoplasmic compartments (Golgi), is cleaved off and free BLI (without GFP) then enters the nucleus in cells outside of the elongation zone. Root picture modified from Barrada et al. (2015). QC: Quiescent center.

In summary, we show here that BLI is the first identified negative regulator of the ER-stress response/UPR in plants. ER-stress responses need to be suppressed during normal plant

development in order to prevent spurious protein degradation. BLI might be an important regulator of ER homeostasis by preventing UPR during plant development and during ER-stress. Moreover, BLI might link epigenetic gene regulation to the ER-stress response in plants, a connection that is not well understood, yet.

Material and Methods

Plant material and growth conditions

Seeds of Columbia-0 (Col-0, N1092), *bli-1* (SAIL_107_D04, N805222), *bli-11* (GABI-Kat_663H12), *bli-2* (SALK_005565, N505565), *bli-3* (SAIL_518_E07, N821933), *clf-28* (SALK_139371, N639371), *bli-1*/*BLI:BLI-GFP* (Kleinmanns et al., 2016 – Manuscript I), and *bli-11*/*BLI:BLI-GFP* (Kleinmanns et al., 2016 – Manuscript I) were sterilized (10 min 70% Ethanol supplemented with 0.05% Triton X-100, 10 min 96% Ethanol) and sown on germination medium (MS; half-strength Murashige and Skoog medium supplemented with 0.5% sucrose, 0.05% MES, and 0.8 % plant agar). Seeds were stratified for two days at 4°C and grown under long day (LD) conditions, (8/16 h dark/light rhythm at 20 °C). *bli-1* and *bli-11* seeds showed a germination delay of two days (Schatlowski et al., 2010), therefore these two genotypes were sown two days earlier than all other genotypes when directly compared, were stratified for two days at 4°C and then transferred to the respective growth condition. For GUS staining, plants were grown for 5 and 12 days on MS under LD conditions.

N. benthamiana leaves were transformed as described in Bleckmann et al. (2010).

Stress experiments

Plants were grown on MS medium for 6 days in LD conditions and then transferred to either MS medium or MS plus 0.3µg/ml Tunicamycin (TM, in DMSO) (SIGMA-ALDRICH), an ER-stress inducer. Plants were grown for additional 6 days in LD conditions and then harvested for RT-qPCR analysis and chlorophyll measurement.

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed as described in Kleinmanns et al., 2016 - Manuscript I

Quantitative PCR

RNA from 12-day-old seedlings was extracted using innuSPEED Plant RNA Kit (Analytik Jena), resuspended in 30 µl RNAse-free water, and treated with DNaseI (Fermentas). cDNA was synthesized from 1 µg RNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific) using Oligo(dT) oligonucleotides. The obtained cDNA was diluted 1:10 and 2 µl of

this dilution were used for RT-qPCR. qPCR was performed in a LightCycler 480 (Roche) using KAPA SYBR FAST qPCR Master Mix according to manufacturer's instructions in a 2-step PCR program (95°C 5:00 min, 40 x (95°C 0:15 min, 60°C 0:30 min)). Expression levels were normalized to AT1G59830 (Czechowski et al., 2005), a gene that does not show expression changes during, or in response to, ER-stress.

Chlorophyll measurement

Samples for chlorophyll measurement were frozen in liquid nitrogen (lN_2) and were ground in presence of lN_2 inside a 1.5 ml reaction tube using micro pistils. 1 ml 100% Acetone was added to the samples. Samples were then vortexed for 10 sec, incubated for 10 min at RT, and then centrifuged 3 min at 16,000 rpm. 800 µl of supernatant were transferred to a new reaction tube and 200 µl H_2O were added to obtain an 80% acetone solution. For detection 200 µl per sample of the pigment extract were used. Absorption of three technical replicates was measured at 646, 663, and 750 nm in a plate reader (BioTek). Total chlorophyll content per mg fresh weight was calculated according to Porra (2002).

GUS staining

GUS staining was performed as described in Kleinmanns et al., 2016 – Manuscript I. Images were taken using a Nikon Stereomicroscope SMZ25.

Imaging

Confocal laser scanning microscopy was performed using LSM 780 and LSM 510 microscopes (Zeiss). Image acquisition was carried out sequentially to prevent crosstalk between channels. GFP was excited at 488 nm, and emission was detected at 510-550 nm. RFP and propidium iodide (PI) were excited at 561 nm and emission was detected at 575-630 nm. Induction of expression in *Arabidopsis* was obtained by inoculation of seedlings with 10 µM ß-estradiol for 12 hours. *N. benthamiana* leaves were brushed with 20 µM beta-estradiol + 0.1% Tween 20, 24 h prior to imaging.

Cloning of mutated constructs

The BLI promoter, including 1.7 kb upstream of the transcriptional start site, was amplified from genomic DNA using primers with ApaI restriction sites (underlined) (F: AGTGGGCCCGAACTGGCAATTCAGAATCGGGAT, R: AGTGGGCCCTGAAAAATACTCGAAATCTCGCAG). ProBLI and pGKGWG (Zhong et al., 2008) were digested with ApaI and re-ligated with T4 DNA ligase (NEB) (=pGKGWG-proBLI). pDONR201_cBLI-STOP (Schatlowski et al., 2010) containing cBLI

without the stop codon, was mutated by Phusion Site-Directed Mutagenesis (Thermo Fisher Scientific; oligonucleotides are shown in (Supplemental Table) and treated with DPNI (Thermo Fisher Scientific). Mutated and non-mutated cBLI (pDONR201_cBLI-STOP) was Gateway cloned into pGKGWG-proBLI (Gateway LR Clonase Enzyme mix, Thermo Fisher Scientific). Mutated cBLI was also cloned into pABindGFP (Bleckmann et al., 2010) for transient expression in *N. benthamiana*.

Supplemental Table 1: Oligonucleotides used for amplification of UPR genes by qRT-PCR.

gene name	ATG number	sequence F	R
bZIP60	AT1G42990	GATGATGACGAAGAAGGAGACG	TCTAACCGCCGCATCTCTAT
bZIP28	AT3G10800	TCCGCATTCAACAGCTCTCT	AACTGGAAAACCTCGGTGCA
BIP3	AT1G09080	GGTGAAGGTGGAGAAGAAACAC	CCTCCGACAGTTTCAATACCGA
NAC103	AT5G64060	CCATTGCTGAGGTCGACATT	ACCACTTAAGATCTCCAGTCCC
NAC089	AT5G22290	AGGCGAAAGAACTGACTGGA	AACCCGGCAAACAACCATAG

Supplemental Table 2: Oligonucleotides used for H3K4me3 ChIP qPCR.
Oligonucleotides binding at the promoter region are labelled as ‘P’ and oligonucleotides binding in the gene body are labelled as ‘B’.

gene name	ATG number	F	R	Reference
SEC31A-P	AT1G18830	GACAACACACAAATGACGTG	GAGAGTGACTCGAAGAAAGC	(Song et al., 2015)
SEC31A-B	AT1G18830	GAACTCGATTTTCAGTCCAA	TTGGATTCCATAAACCGATG	(Song et al., 2015)
NSF-P	AT4G21730	GTCTAGCCAATCAGAGAATG	ACGTACACAAATGTTATGGC	(Song et al., 2015)
NAC103-B	AT5G64060	AACTTGGCACCTGGTTTTCG	AATGTCGACCTCAGCAATGG	
BIP3-P	AT1G09080	TGTCACGTGTCTGCTTGTGA	TAGCCTCGGTAGAGTGTCCT	(Song et al., 2015)
BIP3-B	AT1G09080	CACGGTTCCAGCGTATTTCAAT	ATAAGCTATGGCAGCACCCGTT	(Song et al., 2015)
ERDJ3A-P	AT3G08970	GTGAGTAATTGCCCCTACCA	CTTCCTCTTCTAAGCGTGTC	(Song et al., 2015)
SARA1A-P	AT1G09180	TAAACTCTCCTGGGTCCTGG	ACACGTGGGTAATGGGGACT	(Song et al., 2015)
TIN1-P	AT5G64510	GGCGAAGCCATTGTCAATAC	GGTTTTCACGGGAAGAGATG	(Song et al., 2015)
ACT7	AT5G09810	TAGTGAAAAATGGCCGATGG	CCATTCCAGTTCCATTGTCA	

Supplemental Table 3: Oligonucleotides used for site directed mutagenesis of *cBLI.*

primer name	sequence (mutated nucleotides and NLS tag are underlined)	mutation
BLI-cDNA_mutNLS_MUT	CTTGAGCAGTTTCGTGCTCGAGCTGCAGCAGAAAAAGCT	K(25)A, K(27)A
BLI-cDNA_mutNLS_R	CTTACGGCGTCCAGCTTCAACGTC	
BLI-cDNA_mutNES_MUT	GATTTTTCTAATAGCAAGGCCCGAATAGGTTCATCGAAG	L(116)K, E(117)A, L(118)R
BLI-cDNA_mutNES_R	AACTTTGCCCACTGATTCCTGACC	
NLS-tag cBLI_F	ATGGGGCCCAAGAAAAAGCGCAAGGTTATGGCATCAGCTACTAGTTCCCGG	PKKKRKV (NLS tag)
NLS-tag cBLI_R	AGCCTGCTTTTTTGTACAAACTTGG	

Acknowledgements

J.A.K. and D.S. are supported by a ‘PLUS3’ grant from the Boehringer Ingelheim Foundation.

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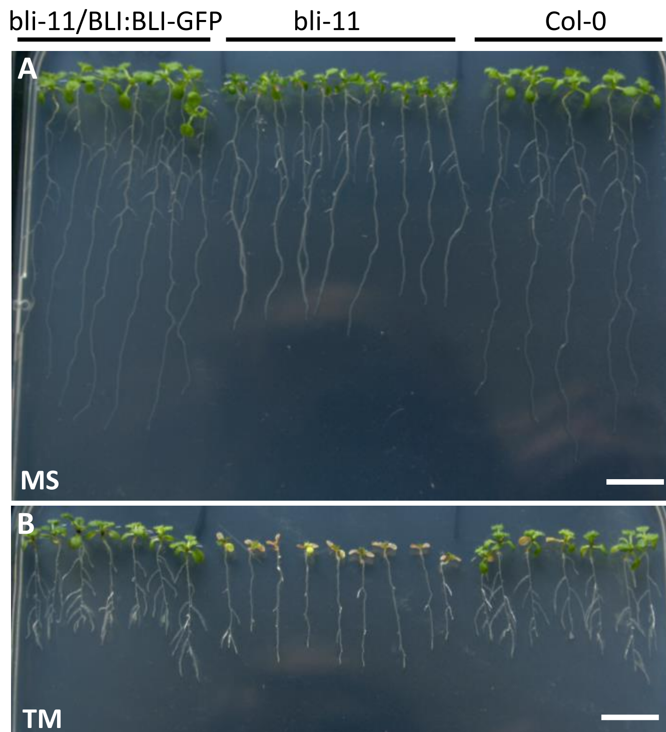
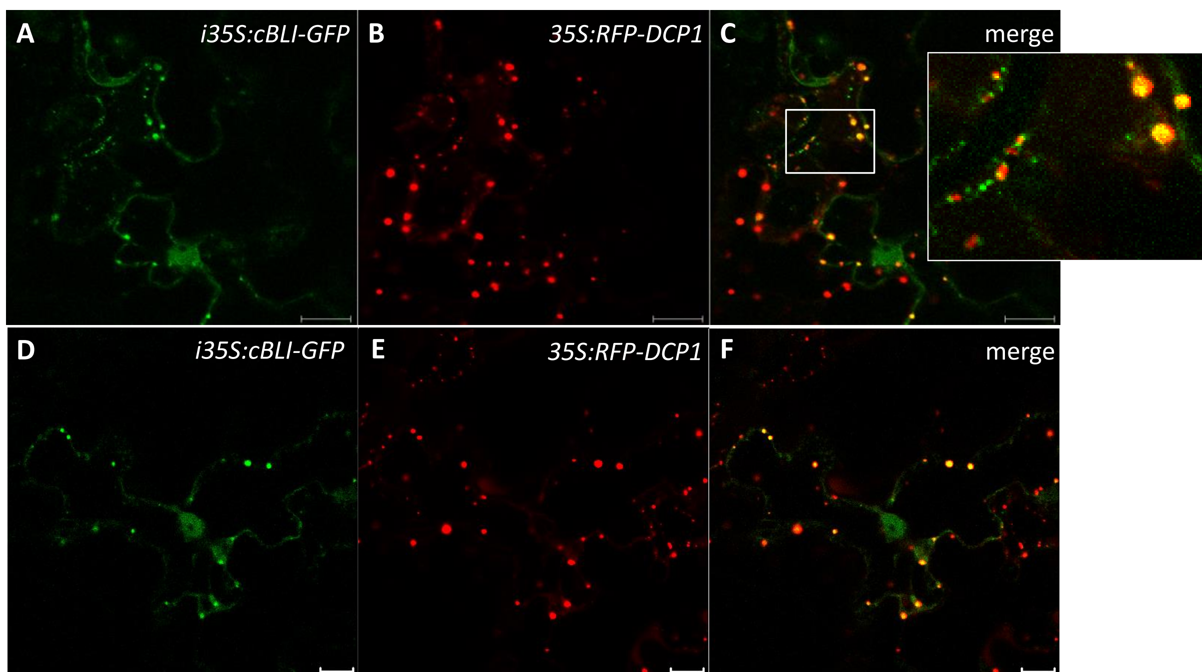
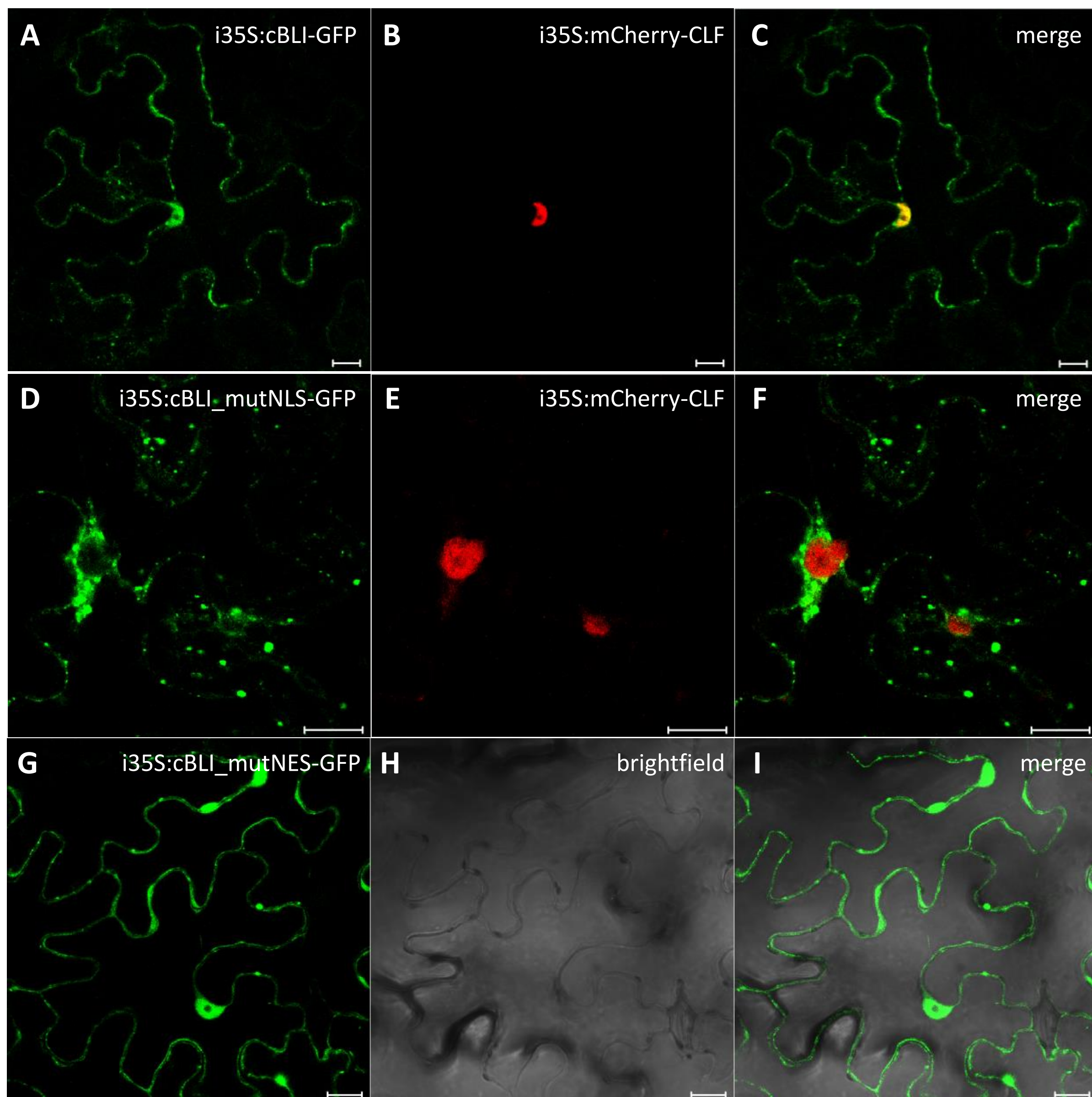
Supplemental information



Supplemental Figure 1: Response of wild type, *bli-11* and *bli-11/BLI:BLI-GFP* to ER-stress induced by Tunicamycin (TM). Seedlings were grown vertically for 6 days on MS medium, transferred to either MS or MS+0.3 µg/ml TM plates and grown for additional 6 days. Like *bli-1* also *bli-11* seedlings became strongly chlorotic during ER-stress treatment. *bli-11* mutants showed a short-root phenotype as is the case for *bli-1*.



Supplemental Figure 2: Expression of *i35S:cBLI-GFP* and *35S: RFP-DCP1* (Moreno et al., 2013), a marker for processing-bodies (p-bodies) in *N. benthamiana* leaf epidermis cells.
Figure A-C and D-F show two different cells expressing BLI-GFP (A, D) and RFP-DCP1 (B, E). Induction of transgene expression by application of 20 µM beta-estradiol 24 h prior to imaging. Scale bars are 20 µm.



Supplemental Figure 3: Expression of mutated *i35S:cBLI-GFP* constructs in *N. benthamiana* leaf epidermis cells. Mutated constructs were co-expressed with *i35S:mCherry-CLF* (B, E), except for *i35S:cBLI-mutNES-GFP* (G-I). Mutation of the BLI NLS led to an altered subcellular localization (D-F), whereas mutation of the NES (G-I) did not alter the subcellular localization of BLI-GFP. Induction of expression by application of 20 µM beta-estradiol 24 h prior to imaging. Scale bars are 20 µm.

4 Concluding Discussion

4.1 BLI is involved in PRC2-dependent epigenetic gene regulation

The plant specific protein BLISTER (BLI) was identified as an interactor of the POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) methyltransferase CURLY LEAF (CLF) (Schatlowski et al., 2010). PRC2 regulates target genes by setting the repressive mark H3K27me3. Approximately 28% of all genes in the *Arabidopsis* genome are targeted by H3K27me3 (Oh et al., 2008). Loss of PRC2 function leads to a strong reduction or even loss of H3K27me3 (Lafos et al., 2011). Strong PRC2 mutants are therefore not able to sustain cell fate decisions, which results in strong developmental and reproductive defects (Chanvivattana et al., 2004; Schubert et al., 2005). The interaction of BLI and CLF indicated that BLI is involved in the regulation of Polycomb group (PcG) target genes, possibly by setting of, or maintaining, PRC2-mediated H3K27me3. However, analysis of several H3K27me3 target genes revealed that the levels of this repressive mark were not changed in *bli-1* mutants, indicating that BLI is not involved in the deposition or maintenance of H3K27me3 (Schatlowski et al., 2010). To understand which H3K27me3 target genes are regulated by BLI, the transcriptional profile of *bli-1* mutants was analyzed. This analysis revealed that a significant number of H3K27me3 target genes was mis-regulated in *bli-1* mutants. Analysis of *bli-1* chromatin showed that H3K27me3 levels, even at the strongest up-regulated PcG target genes, were not changed. These observations further indicate that BLI is important for silencing of H3K27me3 target genes, but not for the deposition or maintenance of this repressive mark. Therefore, BLI likely acts downstream of, or in parallel to, PRC2. *bli-1* was shown to genetically interact with the PRC1 mutant *lhp1* (Schatlowski et al., 2010). In certain PRC1 deficient mutants, H3K27me3 levels at embryo developmental genes were reduced, but were increased at meristem identity and flower developmental genes (Yang et al., 2013). Other PRC1 mutants showed reduced H3K27me3 levels also at other gene categories (Calonje et al., 2008; Derkacheva et al., 2013). The fact that levels of H3K27me3 are neither decreased nor increased in *bli-1* suggests that i) BLI is not involved in PRC2 recruitment, like the PRC1 proteins LHP1 or EMF1 (Calonje et al., 2008; Derkacheva et al., 2013), ii) BLI is not involved in H3K27me3 maintenance, like LHP1 (Derkacheva et al., 2013), and iii) BLI likely has no PRC1 related function since H3K27me3 levels were not increased (Yang et al., 2013). However, the analysis of gene regulation by BLI indicates that BLI belongs to the class of PRC-associated proteins, which are needed to stably silence PcG target genes. Several proteins or protein complexes, which silence genes together with PRC1 or PRC2, were identified in *Arabidopsis*: (VRN-)PHD-PRC2 (De Lucia et al.,

2008), VAL proteins (Yang et al., 2013), ALFIN1-like proteins (AL PHD-PRC1) (Molitor et al., 2014), CYCLOPHILIN71 (CYP71) (Li et al., 2007; Li and Luan, 2011), INCURVATA2 (ICU2) (Barrero et al., 2007; Hyun et al., 2013), and the CUL4-DDB1 ring ubiquitin ligase complex (Dumbliauskas et al., 2011; Pazhouhandeh et al., 2011). These PRC-associated or interacting proteins build a second layer of regulation in the PcG system. This layer is important for proper gene regulation during specific developmental stages and processes, like embryo development, vernalization, or flowering time control, or for the regulation of specific gene categories, like homeotic genes. BLI participates in the PRC2-mediated control of stress-related (ABA-responsive) H3K27me3 target genes, but also regulates gene expression independently of the PcG system.

To understand which target genes are shared by BLI and CLF, I analyzed the commonly mis-regulated genes in *bli-1* and *clf* single, as well as *clf swn* double mutants. Both overlaps were significant, revealing that certain genes are commonly regulated by BLI and CLF/SWN. Importantly, *BLI* was not mis-regulated in *clf* or *clf swn* mutants, and neither *CLF* nor *SWN* were mis-regulated in *bli-1* mutants. This indicates that *BLI* and *CLF/SWN* do not regulate each other's expression. To reveal if BLI affects CLF at the protein level, probably by affecting the localization of CLF inside a cell, the subcellular localization of GFP-CLF was analyzed in *bli* mutants. Increased fluorescence, and hence protein levels, of GFP-CLF were observed in *bli* mutants. Although expression of endogenous *CLF* was unchanged in *bli*, expression of a *35S:GFP-CLF* transgene was strongly increased in *bli-1* mutants, compared to the control. The same was true for GFP-CLF protein levels, as shown by immuno-blot experiments. These data indicate that BLI regulates the expression of the *35S:GFP-CLF* transgene, but not of endogenous *CLF*, and renders the analysis of *CLF* localization in *bli* inconclusive.

The action of PRC2 can be counteracted by Trithorax proteins, which set the activating mark H3K4me3. To understand if the ectopic expression of H3K27me3 target genes in *bli-1* is due to increased levels of activating H3K4me3, the levels of H3K4me3 at several H3K27me3 target genes were analyzed. H3K4me3 levels were only increased at specific genes which are activated by endoplasmic reticulum (ER-) stress (*SEC31A, NAC103*), but not at MADS box TF encoding genes (*SEP2*, *SEP3*). Importantly, gain of H3K4me3 was observed at H3K27me3 target (*SEC31A*) and non-target (*NAC103*) genes. It is to mention, that genes regulated by H3K4me3 were not significantly enriched among mis-regulated genes in *bli-1* and genome wide levels of H3K4me3 were not altered, as indicated by immunoblot analysis. Therefore, BLI is not generally involved in the counteraction of H3K4me3 levels in *Arabidopsis*, but likely

restricts deposition of this mark only at specific, stress-related H3K27me3 target and non-target genes. Importantly, increase of H3K4me3 at *SEC31A* and *NAC103* could also be indirectly regulated by BLI: in the wild *SEC31A* acquired H3K4me3 type in response to ER-stress (Song et al., 2015). Therefore, loss of BLI could induce ER-stress, which would lead to increased levels of H3K4me3 at *SEC31A* and *NAC103* in *bli-1*. BLI would therefore not restrict binding of TrxG proteins to these genes under non-stress conditions, but would regulate transducers of ER-stress responses, which activate *SEC31A* and *NAC103* by recruiting TrxG proteins to these loci in response to ER-stress.

4.2 BLI regulates specific stress responses and developmental regulators in *Arabidopsis*

If a plant experiences stress, extensive changes in gene expression and chromatin modifications, e.g. H3K4me3 and H3K27me3 (Ding et al., 2012; Jaskiewicz et al., 2011; Liu et al., 2014; Sani et al., 2013), are induced in order to properly respond to a given stress. Today, it remains largely unresolved how PcG proteins affect the expression of stress-responsive genes (reviewed in Kleinmanns and Schubert, 2014). The transcriptional profile of *bli-1* mutants revealed that a high number of genes involved in abiotic or biotic stress responses were mis-regulated. Genes responding to drought, heat, high salt, ER-stress, and systemic acquired resistance (SAR), were enriched among up-regulated genes in *bli-1*, indicating that BLI negatively regulates expression of stress-related genes. A principal component analysis (PCA) further revealed that genes mis-regulated in *bli-1* are similarly regulated by drought, wounding, and ER-stress. The involvement of BLI in stress responses was shown before: during cold-stress *bli* mutants failed to induce cold-responsive genes, indicating that BLI positively regulates expression of these genes (Purdy et al., 2010). Transcriptional profiling of *bli-1* revealed that cold-stress responsive genes were enriched among down-regulated genes. This further suggests a positive role of BLI in cold-stress response regulation, which is in contrast to its function in PcG-mediated gene silencing. One explanation for this observation could be that BLI acts as a suppressor of an unknown repressor of cold-responses; therefore, loss of *BLI* would activate the repressor and hence cold-responsive genes could not be properly induced.

Up-regulation of several categories of stress-related genes prompted me to investigate how the loss of *BLI* function affects the plants response to different forms of stress. Therefore, *bli* mutants were subjected to drought and ER-stress. The strong *bli* mutants, *bli-1* and *bli-11*, were hypersensitive to drought and ER-stress. The ectopic activation of stress-related genes during ambient conditions together with the reduced stress tolerance of *bli* mutants indicates that i) BLI is important for the suppression of stress during both, normal growth and stress, and ii) that BLI promotes resistance to stress responses.

ER-stress responses in plants can be caused by extensive gene expression during development, as well as by abiotic and biotic stress (Che et al., 2010; Deng et al., 2011; Gao et al., 2008; Liu et al., 2007b; Liu et al., 2011a; Moreno et al., 2012). In this study, BLI was identified as the first negative regulator of ER-stress responses in plants. A high number of genes responding to ER-stress were up-regulated in *bli-1*, and several of these genes were even stronger expressed in response to ER-stress treatment in the mutant, compared to the wild type. As mentioned above, the analysis of chromatin modifications in *bli-1* revealed that H3K4me3 levels at specific genes were similarly altered in *bli* mutants as in ER-stressed wild type plants (Song et al., 2015). Therefore, these genes could be regulated by ER-stress, which is induced in *bli-1* mutants, rather than by loss of *BLI* function (see above). Analysis of BLIs interaction partners and direct target genes will reveal if BLI directly binds to ER-stress-responsive genes and thereby restricts binding of TrxG proteins.
Responses to cold, drought, and high salt are mediated by the phytohormone abscisic acid (ABA) but also by ABA-independent pathways. Genes regulated by ABA were mis-regulated in *bli-1*. Additionally, a significant number of ABA regulated H3K27me3 target genes was mis-regulated in *bli-1*, revealing that BLI likely acts together with PRC2 to regulate expression of these genes. The role of PcG proteins in drought stress response regulation is only emerging. Like *bli-1*, also *clf* mutants show hypersensitivity to drought stress; additionally, *clf* mutants comprise reduced ABA levels during normal growth and during drought stress (Liu et al., 2014). This indicates that during drought stress ABA-responsive genes might not be properly induced in the *clf* background. Since genes involved in ABA biosynthesis or catabolism, or ABA reception or transport were not mis-regulated in *bli-1*, the reduced drought tolerance is likely due to a different mechanism than in *clf*. However, CLF and BLI are both necessary to cope with drought stress, and probably regulate certain ABA-responsive PcG target genes together.

The strong *clf swn* double mutant completely lacks H3K27me3 (Lafos et al., 2011). Hence, this mutant cannot sustain cell fate decisions during development and develops into a callus-like cell mass early during seedling development (Chanvivattana et al., 2004). The strong *bli* mutants, *bli-1* and *bli-11*, show blister-like structures on several organs, which indicates a loss of cell identity. Moreover, *bli-1* mutants show enhanced endoreduplication and fewer cells, indicating a role for BLI in cell division regulation or cell cycle regulation. GUS reporter assays revealed, that the stem cell marker *CLV3* and the cell division marker *CYCB1;1* showed small domains of ectopic expression in *bli-11* mutants. Therefore, blister-like structures might have meristematic identity, or are undifferentiated, pluripotent cells. The ectopic activity of *CLV3*

and *CYCYB1;1* indicates that BLI is involved in the suppression of these genes in non-native tissues and that BLI is a negative regulator of differentiation by preventing ectopic meristematic activity and endoreduplication without cell division.

4.3 BLI localizes to nuclei and the Golgi in *Arabidopsis* root cells

To analyze the subcellular localization of BLI, BLI-GFP fusion proteins were expressed in *N. benthamiana* leaf epidermis cells (Schatlowski et al., 2010) and *Arabidopsis* root cells (C-terminally truncated BLI) (Purdy et al., 2010). This analysis revealed, that BLI localizes to nuclei and cytoplasmic 'speckles'. In this study, localization of full length BLI-GFP was analyzed in *Arabidopsis* root cells. BLI-GFP was found to localize to nuclei only in cells of the root elongation zone. Co-expression of BLI-GFP and several marker proteins for cytoplasmic compartments revealed that BLI is present at the Golgi in all root cell types. However, BLI-GFP did not colocalize with an ER marker or a marker for the Trans-Golgi network, indicating that BLI is neither secreted nor degraded in a Golgi-dependent manner. BLI does not contain Golgi retention or localization signals, and no myristoylation sites, which could have explained the Golgi association. Likely, BLIs Golgi localization is depending on protein-protein interactions. During ER-stress conditions BLI could interact with bZIP28 at the Golgi. This interaction might partially retain bZIP28 at the Golgi, thereby BLI would regulate the expression of genes activated by bZIP28 during the unfolded protein response (UPR).

Additionally to the colocalization with the Golgi, BLI-GFP also colocalized with a marker for processing-bodies (p-bodies) in *N. benthamiana* leaf epidermis cells. It is tempting to speculate, that gene regulation by BLI could also be achieved by regulation of post-transcriptional processes: p-bodies contain components of the RNAi machinery and other proteins important for degradation and storage of mRNAs (reviewed in Maldonado-Bonilla, 2014). BLI could be involved in these processes, thereby regulating genes independent of the PcG system. If BLI localizes to p-bodies and interacts with its components, or mRNAs in *Arabidopsis,* needs to be analyzed in the future.

The exclusive nuclear localization of BLI-GFP in cells of the root elongation zone raises one important question: how can BLI regulate gene expression if it is not present in the nucleus? It is possible that BLI changes its localization during stress, but preliminary data do not support this hypothesis. Moreover, this scenario can only explain direct gene regulation by BLI during stress, but not during normal development. Transient expression of C-terminally truncated BLI-GFP in *Arabidopsis* revealed that this fusion protein localizes to nuclei but not to cytoplasmic compartments in root cells. It is possible that in cells outside the root elongation zone the BLI

C-terminus, containing GFP, is cleaved off, therefore BLI would be present in nuclei but cannot be detected by confocal microscopy. BLI could hence associate with chromatin in all cell types during development. If cleavage of the BLI C-terminus is occurring *in planta* needs to be analyzed in the future.

4.4 Conclusion

Three main conclusions that can be drawn from this work: i) BLI acts downstream of, or in parallel to, PRC2 in gene repression, without affecting H3K27me3 levels, ii) BLI is a negative regulator of several stress responses, and iii) BLI regulates the expression of certain developmental genes. Therefore, BLI fulfills several functions in *Arabidopsis*: it is involved in the epigenetic gene regulation by the PcG system, it is an important regulator of several stress responses, and it regulates developmental programs. If and how epigenetic gene regulation and stress response regulation by BLI are overlapping was only partially resolved in this study, and needs to be investigated in more detail in the future. However, BLI likely regulates ABA-responsive H3K27me3 target genes together with, or downstream of, PRC2, thereby linking epigenetic gene regulation and stress response regulation.

4.5 Perspectives

To understand the role of BLI in epigenetic gene regulation and in stress response regulation, the analysis of BLIs direct target genes and interaction partners needs to be addressed during normal growth and under stress conditions. To understand which (H3K27me3 target) genes are directly regulated by BLI it will be important to analyze its binding to chromatin. The analysis of BLIs direct interaction partners will reveal if BLI interacts with transducers or executors of (ER-) stress, or with additional Polycomb or Trithorax proteins. Moreover, analysis of its interaction partners will reveal if BLI interacts with Golgi localized proteins, therefore explaining how BLI could be anchored to Golgi vesicles. Furthermore, analysis of BLI interaction partners will reveal if BLI interacts with p-body components and hence is involved in the regulation of mRNAs in the cytoplasm. *BLI* expression is highly increased in response to heat-stress (Sullivan et al., 2014) but no other form of stress. Therefore, BLIs role in this particular stress response needs to be further analyzed. By analyzing the role of BLI in the regulation of stress-related H3K27me3 target genes we will learn more on how epigenetic modifications and stress response regulation are connected in plants.

One important question that needs to be answered in the future is how BLI can regulate gene expression when it localizes to nuclei only in a few cells. Immunostaining experiments, using anti-BLI antibody, analysis of N-terminal GFP fusion proteins, and expression of C-terminally

truncated BLI will reveal if BLI localizes to nuclei outside the root elongation zone. To understand which BLI domain is responsible for its Golgi association it will be important to delete or mutate the BLI C-terminal coiled coil domain as C-terminally truncated BLI-GFP did not localize to cytoplasmic compartments. ER-stress transducers change their localization in response to stress. To understand whether this is also true for BLI its subcellular localization needs to be analyzed during normal growth and under stress conditions.

In summary, BLI is a protein with diverse functions: it is involved in PcG-dependent epigenetic gene regulation, it is an important stress response regulator, it regulates certain developmental programs, and it maintains cell identity. Further analysis of BLI target genes, interaction partners and its subcellular localization is required to comprehensively understand how BLI regulates gene expression together with PRC2, and independently, during development and in response to stress.

5 Abstract

5.1 Abstract

BLISTER (BLI) is a plant specific Protein which interacts with the PRC2 (POLYCOMB REPRESSIVE COMPLEX 2) methyltransferase CLF (CURLY LEAF). PRC2 is highly conserved among animals and plants and represses thousands of genes by trimethylation of histone 3 lysine 27 (H3K27me3). PRC2-mediated H3K27 trimethylation is not sufficient for gene silencing (Schubert et al., 2006); additional proteins are required for stable repression of certain H3K27me3 target genes. This study aimed to elucidate whether BLI regulates the expression of a specific class of H3K27me3 target genes, and whether it has PRC2 related and unrelated functions. Therefore, the transcriptional profile of plants deficient in *BLI* gene function (*bli-1* mutant) was analyzed to determine BLIs target genes. This analysis revealed that a high number of Polycomb group (PcG) protein target genes was mis-regulated in *bli-1*. Interestingly, the levels of H3K27me3 at PcG target genes remained unaffected in *bli-1* mutants, indicating that BLI acts downstream of, or in parallel to PRC2 in H3K27me3-dependent gene silencing.

Furthermore, the analysis of the transcriptional profile revealed that a high number of genes responding to drought, heat, high salt, endoplasmic reticulum (ER-) stress, and systemic acquired resistance (SAR) was mis-regulated in *bli-1*. Additionally, genes regulated by the plant hormone abscisic acid (ABA), but no key regulators of ABA biosynthesis or catabolism, or ABA reception or transport, were mis-regulated, indicating that downstream ABA responses are affected in *bli-1*. *bli* mutants showed increased susceptibility towards drought and ER-stress treatment, indicating that BLI is a negative regulator of stress responses in plants. The up-regulation of ER-stress-responsive genes in *bli-1* together with the increased sensitivity towards ER-stress treatment shows that BLI is the first identified negative regulator of ER-stress responses in plants. *bli-1* mutants showed increased levels of H3K4me3, an activating histone modification, on several ER-stress responsive genes. This indicates that under normal growth conditions BLI might restrict H3K4me3 at stress responsive genes.

The analysis of the subcellular localization of BLI-GFP fusion proteins revealed that BLI is dual localized. BLI-GFP was present in the nuclei of cells of the root elongation zone, but not in more differentiated cells, or cells of the root tip, and it localized to the Golgi in all cell types. BLI-GFP did not colocalize with marker proteins for the ER or the Trans-Golgi network, indicating that BLI is neither secreted nor degraded in a Golgi-dependent manner. The localization of BLI in cytoplasmic compartments is likely dependent on its C-terminus.

Strikingly, neither the mutation of BLIs NES (nuclear export signal) nor the addition of a second, strong NLS (nuclear localization signal) could stably force BLI to localize to the nucleus or could trap it there, revealing a second layer of BLI regulation independent from its nuclear import and export signal.

In summary, this study shows that BLI regulates a high number of H3K27me3 target genes, without affecting H3K27me3 levels. Therefore, BLI is an important regulator of H3K27me3 target gene expression downstream of, or in parallel to, PRC2. This work hence further shows that PRC2-interactors are required for the stable repression of certain genes in plants. Therefore, the analysis of BLI function in H3K27me3-dependent target gene silencing contributes to our understanding of the epigenetic gene regulation in plants. Additionally, this work revealed that BLI is involved in the regulation of specific stress responses in *Arabidopsis*. Although stress response regulation by BLI is partially independent of PRC2, BLI could link the regulation of stress responses to the PcG system and epigenetic gene regulation.

5.2 Zusammenfassung

BLISTER (BLI) ist ein pflanzenspezifisches Protein, das mit der PRC2 (POLYCOMB REPRESSIVE COMPLEX 2) Methyltransferase CLF (CURLY LEAF) interagiert. PRC2 ist in Tieren und Pflanzen stark konserviert und unterdrückt die Expression tausender Gene durch Trimethylierung von Lysin 27 an Histon 3 (H3K27me3). PRC2-vermittelte H3K27 Trimethylierung ist nicht ausreichend für die Genstillegung (Schubert et al., 2006); weitere Proteine sind von Nöten um die Expression von H3K27me3 Zielgenen stabil zu unterdrücken. Diese Studie hatte zum Ziel aufzudecken ob BLI die Expression bestimmter H3K27me3 Zielgene reguliert, und ob es PRC2 abhängige und unabhängige Funktionen besitzt. Um die Zielgene von BLI zu bestimmen wurde in dieser Studie das Transkriptionsprofil von Pflanzen analysiert, die kein funktionstüchtiges *BLI* Gen enthalten (*bli-1* Mutante). Diese Analyse ergab, dass in *bli-1* eine hohe Anzahl von Genen fehlreguliert ist, die von Polycomb Gruppen (PcG) Proteinen unterdrückt werden. Interessanterweise konnten keine Änderungen in der H3K27me3 Menge an PcG Zielgenen in *bli-1* festgestellt werde, was darauf hindeutet, dass BLI die Stilllegung von Genen zeitlich nach PRC2 vermittelter H3K27 Trimethylierung steuert, oder parallel dazu.

Die Analyse des Transkriptionsprofils ergab außerdem, dass in *bli-1* eine hohe Anzahl von Stress regulierten Genen fehlreguliert ist: Gene, die in die Antwort auf Trocken-, Hitze-, Salz-, und Endoplasmatisches Retikulum (ER-) Stress involviert sind und solche die durch ‚systemisch erworbene Resistenz' (SAR) reguliert werden, waren in *bli-1* fehlreguliert. Darüber hinaus waren in *bli-1* Gene fehlreguliert, die vom Pflanzenhormon Abscisinsäure (ABA) reguliert werden, jedoch nicht in deren Aufbau, Abbau, Perzeption oder Transport involviert sind. Dies deutet darauf hin, dass in *bli-1* Pflanzen ABA-nachgeschaltete Antworten fehlreguliert werden. *bli* Mutanten zeigten eine gesteigerte Sensibilität wenn sie ER- oder Trockenstress ausgesetzt wurden. Dies deutet darauf hin, dass BLI ein negativer Regulator von Stressantworten in Pflanzen ist. Die Überexpression von ER-Stress aktivierten Genen zusammen mit der verminderten Toleranz von *bli* Mutanten gegenüber ER-Stress deutet darauf hin, dass BLI der erste identifizierte negative Regulator von ER-Stress in Pflanzen ist. *bli-1* Mutanten zeigten gesteigerte Mengen an H3K4me3, einer aktivierenden Histonmodifikation, an einigen ER-Stress Genen. Eines dieser Gene war ein PcG Zielgen, was darauf hindeutet, dass BLI unter normalen Wachstumsbedingungen die Trimethylierung von H3K4 an Stress-assoziierten PcG Zielgenen und Nicht-Zielgenen verhindert. Die Analyse der subzellulären Lokalisation von BLI-GFP Fusionsproteinen ergab, dass BLI eine duale Lokalisation aufweist. BLI-GFP war in den Zellkernen von Wurzelzellen in der Zellstreckungszone vorhanden, jedoch

nicht in Kernen von differenzierten Wurzelzellen oder Zellen der Wurzelspitze. Außerdem kolokalisierte BLI-GFP mit dem Golgi in allen Zelltypen. Die BLI-GFP Lokalisation korrelierte jedoch nicht mit dem ER oder Proteinen die das Trans-Golgi Netzwerk markieren, was auf eine Golgi-abhängige Sekretion oder Degradation von BLI hätte hinweisen können. Die Lokalisation von BLI-GFP in cytoplasmatischen Kompartimenten ist wahrscheinlich vom BLI C-terminus abhängig. Bemerkenswerterweise führten weder die Mutation der BLI Kernexportsequenz (NES) noch der Anhang einer zweiten, starken Kernlokalisierungssequenz (NLS) dazu, dass BLI stabil im Kern vorhanden war. Dies lässt auf eine weitere Regulationsebene von BLI, unabhängig von seiner NLS und NES, schließen.

Zusammenfassend zeigt diese Studie, dass BLI eine hohe Anzahl von H3K27me3 Zielgenen reguliert ohne die Menge an H3K27me3 an diesen Genen zu verändern. Daher ist BLI ein wichtiger Regulator von H3K27me3 Zielgenen der zusammen mit PRC2, oder zeitlich danach, diese Gene reguliert. Diese Studie bestätigt somit, dass Interaktoren von PRC2 wichtig sind um die Expression bestimmter Gene in Pflanzen zu unterdrücken. Daher trägt die Analyse der Funktionsweise von BLI in der H3K27me3-vermittelten Genstillegung dazu bei die epigenetische Genregulation in Pflanzen zu verstehen. Außerdem wurde in dieser Studie gezeigt, dass BLI spezifische Stressantworten in *Arabidopsis* reguliert. Obwohl BLI Stressantworten zum Teil unabhängig von PRC2 reguliert, könnte BLI dennoch Stressantworten mit dem PcG System und epigenetischer Genregulation verbinden.

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7 Appendix

7.1 Abbreviations

°C	Degree Celsius	µg	microgram
A. thaliana	*Arabidopsis thaliana*	µl	microliter
A. tumefaciens	*Agrobacterium tumefaciens*	NES	nuclear export signal
bp	Base pair	ng	nanogram
Col	Ecotype Columbia	NLS	nuclear localization signal
ChIP	Chromatin immunoprecipitation	nm	nanometer
dH_2O	distilled water	nos	Nopaline Synthase
DNA	Deoxyribonucleic acid	PCR	Polymerase chain reaction
E. coli	*Escherichia coli*	PI	Propidium iodide
EtOH	Ethanol	Pro	Promoter
ER	Endoplasmic reticulum	PcG	Polycomb group
Gent	Gentamycin	PPT	Phosphinothricin
GFP	Green fluorescent protein	qRT-PCR	quantitative Reverse Transcriptase PCR
H	hour		
HRP	horseradish peroxidase	Rif	Rifampicin
Kan	Kanamycin	RNA	Ribonucleic acid
L	liter	Sulf	Sulfadiazin
M	molar	T-DNA	Transfer-DNA
mg	milligram	TM	Tunicamycin
ml	milliliter	TrxG	Trithorax group
mM	millimolar	w/v	weight per volume
MS	Murashige & Skoog Medium	wt	wild type

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Danksagung

Mein Dank gilt besonders meinem Doktorvater Prof. Dr. Daniel Schubert der mir dieses spannende Projekt übertragen hat. Vielen Dank für deine Ideen, Hilfestellung und die zahlreichen Diskussionen die zum Gelingen dieser Arbeit unerlässlich waren!

Weiterhin möchte ich mich bei meinem Mentor Prof. Dr. Rüdiger Simon bedanken für die Übernahme des Zweitgutachtens und die hilfreichen Diskussionen die zum Gelingen dieser Arbeit beigetragen haben.

Ich danke auch meinen iGRAD Komiteemitgliedern Prof. Dr. Michael Feldbrügge und Prof. Dr. Ute Höcker für die hilfreichen Diskussionen.

Ein herzliches Dankeschön auch an meine Laborkollegen die mich während meiner Zeit in Düsseldorf begleitet und durch Hilfestellung und Diskussionsbereitschaft unterstützt haben. Liebe Mareike, Marcel, Nora, Maurice, Gregor, Sara, Yu, Pawel, Inês, Olga und Jenny vielen Dank für die schöne Zeit und die vielen lustigen Momente mit euch!

Vielen Dank auch an die ‚Simons‘, die mir immer hilfreich zur Seite standen und die meine Zeit in Düsseldorf bereichert haben.

Ein herzliches Dankeschön auch an die iGRAD Koodinatorinnen Sigrun Wegener-Feldbrügge und Petra Fackendahl für Organisation, Hilfestellung und die Erinnerungen an wichtige Fristen und Termine.

Außerdem möchte ich den iGRAD Mitgliedern danken, die mir immer hilfreich zur Seite standen und durch Fragen und Diskussionen meine Arbeit bereichert haben.

Vielen Dank auch den tollen Freunden die ich in Düsseldorf kennenlernen durfte und die mit mir durch Dick und Dünn gegangen sind. Isa, Rajen, Vivi, Lea, Rudi, Tobi und Pia, danke für die schöne Zeit in der ‚Feindstadt‘!

Meiner Familie gebührt der Größte Dank. Danke, dass ihr immer für mich da wart und mir in den schönen sowie den schlimmen Stunden zur Seite standet. Papa, du bist nicht mehr hier um diesen Moment mit mir zu teilen, ich weiß aber, dass du sehr stolz auf mich wärest. Danke für deine Unterstützung die du mir auch zuletzt noch gegeben hast!

Ohne dich geht es nicht… David, du bist und warst immer an meiner Seite. Du hast mir immer wieder Mut gemacht wenn es aussichtslos erschien und hast dich mit mir gefreut wenn es super lief. Danke, dass du in meinem Leben bist! Und für die Korrektur!