GLUCAN SYNTHASE-LIKE 8: A Key Player in Early Seedling Development in Arabidopsis

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ABSTRACT

Plants’ cell walls have unique chemical composition and features which enable them to play essential roles during plant development as shaping the cells and providing intercellular communication between adjacent cells. Polysaccharides, including callose, and glycoproteins are known as the main constituents of the cell wall. Callose, a linear β-1,3-glucan polymer, is accumulated at the cell plate during cytokinesis, in plasmodesmata, where it regulates cell-to-cell communication, in dormant phloem, where it seals sieve plates after mechanical injury and pathogen attack, and in male and female gametophytes. *GLUCAN SYNTHASE-LIKE* (*GSL*) genes in Arabidopsis comprise a family of 12 members. A new allele of *GSL8*, *essp8*, was identified as having seedling-lethal phenotype through a genetic screen for Arabidopsis mutants showing ectopic expression of seed storage proteins (*essp*). The gene responsible for the observed mutant phenotype was detected using a combination of bulked-segregant analysis, rough-mapping, and next-generation mapping. An EMS-induced point mutation was identified at an intron splice site of *GSL8*, predicted to introduce a premature STOP-codon. *essp8* seedlings exhibit pleiotropic phenotypic defects, including disruption of root tissue patterning, dwarfism and seedling lethality. Histochemical detection of callose and cell-to-cell diffusion assays showed reduction of callose deposition at the cell plates and plasmodesmata, cytokinesis defects and significant increase in size exclusion limit of plasmodesmata in *essp8* seedlings. Further investigation showed that the increase in size exclusion limit leads to an alteration in symplastic trafficking in primary roots of *essp8* seedlings. Plasmodesmata defects in *essp8* induce ectopic movement of two non-cell-autonomous factors, SHORT ROOT and microRNA165/6, both required for root radial patterning during embryonic root development. Attempts to identify the components of a hypothetical callose synthase complex revealed the interaction of *GSL8* with two plasmodesmata-associated proteins, PLASMODESMATA-LOCALIZED PROTEIN 5 and β-1,3-GLUCANASE, as well as SUCROSE SYNTHASE 1, suggesting that they all might be parts of a single complex. The proposed putative complex might regulate callose deposition at the plasmodesmata and thereby determines the size exclusion limit. In summary, my findings suggest that GSL8 is required for cell wall integrity, maintaining the basic ploidy level and regulation of symplastic movement during early seedling development in Arabidopsis.
KEYWORDS

*Arabidopsis thaliana*, callose, GLUCAN SYNTHASE-LIKE 8, cytokinesis, plasmodesmata, β-1,3-GLUCANASE, PLASMODESMA-LOCALIZED PROTEIN 5, intercellular signaling, symplastic trafficking, callose synthase complex
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# TABLE OF CONTENTS

ABSTRACT............................................................................................................................... ii

KEYWORDS................................................................................................................................... iii

ACKNOWLEDGMENTS ................................................................................................................. iv

TABLE OF CONTENTS ................................................................................................................ v

LIST OF TABLES ........................................................................................................................ xi

LIST OF FIGURES ..................................................................................................................... xii

LIST OF APPENDICES ................................................................................................................ xv

LIST OF ABBREVIATIONS ........................................................................................................ xvi

CHAPTER 1 ...................................................................................................................................... 1

1 INTRODUCTION .................................................................................................................... 2

1.1 Plant cell wall: more than a physical barrier ................................................................. 2

1.1.1 Callose ............................................................................................................................. 2

1.1.2 Structure of callose ......................................................................................................... 2

1.1.3 Callose in fungi ................................................................................................................ 4

1.1.4 Callose in plants .............................................................................................................. 4

1.1.5 Role of callose in cytokinesis and cell plate formation .............................................. 5

1.1.6 Role of callose in plasmodesmata regulation ............................................................. 7

1.2 Callose biosynthesis .......................................................................................................... 9

1.2.1 Glucan synthase in fungi .............................................................................................. 9
1.2.2 Glucan synthase-like proteins in plants ........................................... 10
1.2.3 GLUCAN SYTHASE-LIKE 8 .............................................................. 13
1.2.4 Callose synthase complex ............................................................... 15
1.3 Regulatory role of plasmodesmata in development ............................. 16
1.4 Protein constituents of plasmodesmata ............................................. 17
  1.4.1 Plasmodesmata-localized proteins .................................................. 20
  1.4.2 β-1,3-Glucanases ........................................................................... 21
1.5 Symplastic movement of mobile factors through plasmodesmata ......... 22
  1.5.1 Mobile transcription factors ............................................................ 23
  1.5.2 SHORT ROOT ................................................................................ 24
  1.5.3 WUSCHEL ...................................................................................... 25
  1.5.4 Movement of small RNAs through plasmodesmata ........................ 27
1.6 Regulation of plasmodesmata size exclusion limit ................................ 28
1.7 Dynamic modulation of plasmodesmata connectivity ........................... 30
1.8 Identification of the essp8 mutant ....................................................... 31
1.9 Thesis objectives .................................................................................. 32

CHAPTER 2 .................................................................................................. 33

2 MATERIAL AND METHODS ....................................................................... 34
  2.1 Plant materials and growth conditions ............................................... 34
  2.2 Crossing of Arabidopsis plants ............................................................. 34
2.3 Histochemical assays .................................................................................................. 35
  2.3.1 GUS staining ....................................................................................................... 35
  2.3.2 Sudan red staining ............................................................................................... 35
  2.3.3 Aniline blue staining ......................................................................................... 35
  2.3.4 Propidium iodide staining .................................................................................. 36
2.4 Next-generation mapping of essp8 .............................................................................. 36
2.5 Plasmodesmata size exclusion limit assay ................................................................. 37
2.6 Generation of stable transgenic plants ...................................................................... 37
  2.6.1 Generation of transgenic constructs ................................................................... 38
  2.6.2 Generation of centromere-labeled transgenic plants ........................................ 39
  2.6.3 Generation of miRNA165/6-sensor line ........................................................... 39
  2.6.4 Generation of artificial miRNA transgene constructs ....................................... 40
2.7 Polymerase chain reaction-based genotyping ............................................................ 40
2.8 Gene expression analysis .......................................................................................... 41
2.9 Förster resonance energy transfer ............................................................................. 41
2.10 Microscopy and image analysis ............................................................................... 42
2.11 Statistical analysis ................................................................................................... 42
CHAPTER 3 ..................................................................................................................... 43
3 RESULTS ...................................................................................................................... 44
  3.1 Map-based cloning of the essp8 mutation .............................................................. 44
3.1.1 Morphological phenotype of \textit{essp8} seedlings ........................................44
3.1.2 Next-generation mapping of \textit{essp8} locus ........................................50
3.1.3 \textit{essp8} is allelic to \textit{GSL8} .................................................................55
3.2 Phylogenetic analysis of Arabidopsis GSLs ..................................................59
  3.2.1 The cytoplasmic domains of Arabidopsis GSLs are highly conserved .... 59
3.3 Callose deposition at both cell plate and PD is decreased in \textit{essp8} root ....61
3.4 \textit{essp8} is a cytokinesis-defective mutant ................................................61
  3.4.1 \textit{GSL8} is required for the completion of plant cytokinesis ............... 61
  3.4.2 Cytokinesis impairment in \textit{essp8} induces ectopic endomitosis .......... 65
3.5 \textit{GSL8} regulates symplastic connectivity through plasmodesmata .......... 68
  3.5.1 Loss of \textit{GSL8} induces an increase in plasmodesmata size exclusion limit .... 68
  3.5.2 Dysregulation of \textit{SHORT ROOT} movement through PD in the \textit{essp8} roots .............................................................. 72
  3.5.3 The movement of miR165/6 is altered in the \textit{essp8} root ............... 76
  3.5.4 WUSCHEL symplastic movement might be dysregulated in \textit{essp8} shoot apical meristem .............................................................. 76
3.6 Attempts to rescue \textit{essp8} mutant phenotype ..................................78
  3.6.1 Knocking out \textit{AtBG_PPAP} cannot restore callose balance at the PD in \textit{essp8} ................................................................. 78
  3.6.2 \textit{PDLP5} overexpression induces callose deposition at PD ............... 81
3.6.3 PDLP5 and AtBG_PPAP regulate PD’s SEL .......................................................... 83

3.7 Identification of GSL8 interacting partners .......................................................... 83

3.7.1 GSL8 cytoplasmic domain is required for its interaction with SUS1, AtBG_PPAP, PDLP5 and SCD1 .......................................................... 89

3.7.2 GSL10 might be part of the callose synthase complex ........................................ 89

CHAPTER 4 .................................................................................................................. 93

4 DISCUSSION ............................................................................................................. 94

4.1 essp8 is a new allele of GSL8 .............................................................................. 94

4.1.1 gsl8 mutation causes embryo defects and seedling lethality ......................... 95

4.2 gsl8 phenotypic defects are partially caused by cytokinesis impairment ............. 96

4.2.1 Cytokinesis defects might cause lethality of essp8 seedlings ......................... 97

4.2.2 Cytokinesis-defective mutants do not exhibit severe tissue impairments 97

4.3 Cell-to-cell communication is relaxed in essp8 hypocotyls ............................... 99

4.3.1 GSL8 is required for highly-regulated trafficking of SHR ......................... 100

4.3.2 Loss of GSL8 dysregulates symplastic movement of microR165/6 .............. 102

4.4 PDLP5 function is likely to be GSL8-dependent ................................................. 103

4.5 GSL8 forms a complex to synthesize callose and regulate its deposition ........... 106

4.5.1 SUCROSE SYNTHASE 1, a provider of UDP-Glc to GSL8 ....................... 106

4.5.2 A PD-localized β-1,3-glucanase and PDLP5 regulate PD through GSL8 .......... 107

4.5.3 SCD1, an interacting partner of GSL8 ......................................................... 108
4.5.4 GSL8 and GSL10 interplay might be required for proper function of callose synthase complex................................................................. 108

4.5.5 GSL8 cytoplasmic loops are required for its interaction with the partners.......................................................................................... 109

PERSPECTIVES ........................................................................................................ 111

BIBLIOGRAPHY ...................................................................................................... 113

CURRICULUM VITAE ................................................................................................ 155
LIST OF TABLES

Table 1.1 List of a subset of proteins previously identified in the PD proteome. .......... 18

Table 3.1 Segregation of homozygous essp8 seedlings in the progeny of selfed ESSP8/essp8 heterozygous plants................................................................. 46

Table 3.2 The percentage of defective seeds in one silique from selfed ESSP8/essp8 heterozygous plants................................................................. 47
LIST OF FIGURES

Figure 1.1  Chemical structure of callose. ................................................................. 3

Figure 1.2  Formation of defective cell plate can lead to abnormal mitosis. ............... 6

Figure 1.3  Diagram of a simple primary PD............................................................... 8

Figure 1.4  Diagram of SHR and miR165/6 intercellular movement in Arabidopsis root. .............................................................................................................................. 26

Figure 1.5  Callose deposition at the neck region regulates PD permeability. ............. 29

Figure 3.1  Morphological phenotype of the essp8 mutant. .................................... 45

Figure 3.2  The essp8 mutation causes somatic embryo formation with an incomplete penetrance. ............................................................................................................ 48

Figure 3.3  essp8 mutant seedlings form somatic embryos. ..................................... 49

Figure 3.4  Rough mapping of the essp8 mutation. .................................................. 51

Figure 3.5  Identification of the essp8 mutation using NGM. ................................... 52

Figure 3.6  Detection of SNPs within the candidate region for the essp8 mutation..... 53

Figure 3.7  GLUCAN SYNTHASE-LIKE 8 (GSL8) genomic and protein structure..... 54

Figure 3.8  Morphological phenotypes of gsl8 T-DNA mutant seedlings compared to essp8........................................................................................................................................ 56

Figure 3.9  Analysis of the root morphological phenotype of gsl8 mutants showing severe defects in root tissue patterning................................................................. 57

Figure 3.10 Allelism test confirms that ESSP8 is an allele of GSL8. ....................... 58

Figure 3.11 Phylogenetic tree of Arabidopsis GSLs. .................................................. 60
Figure 3.12  The predicted conserved domains of the GSL8 protein. ......................... 62

Figure 3.13  Callose deposition in the primary roots of gsl8 mutants. ....................... 63

Figure 3.14  Morphological phenotypes of cytokinesis-defective mutants. ............... 66

Figure 3.15  Formation of binucleated cells in the primary roots of gsl8 and cytokinesis-
defective mutants. ................................................................. 67

Figure 3.16  Comparison of centromere numbers in the primary roots of wild-type
Col-0 and gsl8 mutants shows an increase in gsl8 mutants. ....................... 69

Figure 3.17  Comparison of polyploid cells in essp8 mutant at different ages shows an
increase in ploidy level with age ...................................................... 71

Figure 3.18  Quantitative measurement of the fluorescent probes movement in essp8
hypocotyl ...................................................................................... 73

Figure 3.19  Symplastic movement of SHR is altered in essp8 ..................................... 75

Figure 3.20  Cell-to-cell movement of miR165/6 is dysregulated in essp8 .................. 77

Figure 3.21  Analysis of CLAVATA3 transcript levels by qRT-PCR. ......................... 79

Figure 3.22  Phenotypes of atbg-1 gsl8 double mutants ........................................... 82

Figure 3.23  Phenotype of PDL5-OE seedlings and PDL5 subcellular localization... 84

Figure 3.24  Measurement of symplastic movement in various genetic and transgenic
backgrounds. ............................................................................. 85

Figure 3.25  A hypothetical interactome network for GSL8. .................................... 87

Figure 3.26  GSL8 interacts with sucrose synthase, AtBG_PPAP, PDL5 and SCD1... 88

Figure 3.27  The GSL8 cytoplasmic loop is required for its interaction with SUS1,
AtBG_PPAP, PDL5 and SCD1. ...................................................... 90
Figure 3.28  Morphological phenotype of XVE::aMIRGSL8/GSL10 seedling.............. 92

Figure 4.1  A proposed model for GSL8 role in PD regulation................................. 110
LIST OF APPENDICES

Appendix I  List of mutant lines and primers used for genotyping ......................... 138
Appendix II List of primers used for *essp*8 rough-mapping ................................. 140
Appendix III List of primers used to generate expression, overexpression and FRET-related constructs .................................................. 141
Appendix IV T-DNA insertions-specific primers used for genotyping ..................... 142
Appendix V List of primers used for qRT-PCR..................................................... 143
Appendix VI List of Arabidopsis GSLs accession numbers .................................... 144
Appendix VII Alignment of the Arabidopsis GSL family amino acid sequence .......... 145
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>amiRNA</td>
<td>Artificial microRNA</td>
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<td>APETALA 2</td>
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<td>Bacterial Artificial Chromosome</td>
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<td>BG</td>
<td>Endo-1,3-β-GLUCOSIDASE</td>
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<td>bHLH</td>
<td>Basic Helix-Loop-Helix</td>
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<td>BSA</td>
<td>Bulked-Segregant Analysis</td>
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<td>Callose Synthase</td>
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<td>CaMV</td>
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<td>cDNA</td>
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<tr>
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<td>CEI</td>
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<tr>
<td>cM</td>
<td>Centimorgan</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>dsRNAi</td>
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<td>EDTA</td>
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<td>essp</td>
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<td>FRET</td>
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<td>FT</td>
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<td>TVCV</td>
<td>Turnip Vein Clearing Virus</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>Uracil-Diphosphate Glucose</td>
</tr>
<tr>
<td>UGT1</td>
<td>UDP Glucose Transferase 1</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
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<tr>
<td>VRC</td>
<td>Viral Replication Complex</td>
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<td>WT</td>
<td>Wild Type</td>
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<tr>
<td>WUS</td>
<td>WUSCHEL</td>
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<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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All numerical units included in this thesis are standard SI units.
1 INTRODUCTION

1.1 Plant cell wall: more than a physical barrier

Proper development of multicellular organisms is achieved through coordination of cell proliferation and differentiation. Different from animal systems, plant cells have rigid cell walls and, therefore, remain still after cell division. Plant cell walls have a unique chemical composition that enables them to perform several essential functions during plant development, such as shaping different types of cells, providing intercellular communication between adjacent cells and serving as a source of signaling molecules (Burton et al., 2010). Specialized cell walls in plants are responsible for shaping the cells. Plant cell walls are mainly composed of polysaccharides such as cellulose and callose, and glycoproteins (Lerouxel et al., 2006). During the past two decades, due to the emergence of new cellular, molecular and genomics tools, an impressive progress has been made towards identification of callose structure and its important roles in plant development.

1.1.1 Callose

The chemical structure of callose was characterized for the first time in 1957 by Aspinall and Kessler (1957). Callose is formed by β-glucan polysaccharides and known as a component of cell walls in yeasts, plants, fungi, and lichens. β-glucans usually form local depositions at the cell wall and can also act as an energy reservoir in living organisms (Piršelová and Matušíková, 2013).

1.1.2 Structure of callose

Callose is a linear β-1,3-glucan with some 1,6 branches formed by hundreds of glucose residues bound by β-1,3-glycosidic bridges (Fig. 1.1). The structure and chemical composition of callose, such as the number of branches, can differ slightly depending on the cell wall type. The callose structure is not well characterized compared to cellulose, which is a linear long (>1,000 glucose units) chain of unbranched β-1,4-glucan with a crystal-like structure. Callose seems to form more of a helical structure (Piršelová and Matušíková, 2013).
**Figure 1.1** Chemical structure of callose.

Callose is a polymer of glucose formed by hundreds of glucose residues bound via β-1,3-glycosidic bridges. A Few 1,6 branches can also be found in callose structure. Figure modified from Chen and Kim (2009).
1.1.3 Callose in fungi

Glucan is known as the major structural polysaccharide in the cell wall of fungi which constitutes approximately 50-60% of its dry weight (Kapteyn et al., 1999). Glucan polymers are formed through assembly of repeating glucose residues into chains using different types of chemical linkages. Around 65-90% of the fungi cell wall glucan is β-1,3-glucan, with a lower ratio of other glucans, such as β-1,6-, mixed β-1,3- and α-1,4-, α-1,3-, and -1,4-linked glucans (Bernard and Latgé, 2001; Klis et al., 2001; Grün et al., 2005). β-1,3-glucan has been characterized as the most important component of fungi cell walls which forms a platform for the other components to attach covalently. Therefore, β-1,3-glucan synthesis is required for cell wall formation and normal development of fungi (Bowman and Free, 2006). Polymers of β-1,3-glucan are synthesized by β-1,3-glucan synthase complexes, which are associated with the plasma membrane. β-1,3-glucan polymers are expelled into the extracellular space after synthesis (Shematek et al., 1980; Douglas, 2001). Glucan chains are integrated within the cell wall at points of active cell wall synthesis.

Glucan synthase complexes are mainly localized to areas of cell growth, budding, or branching (Qadota et al., 1996; Beauvais et al., 2001). Long linear chains of glucan have approximately 1,500 glucose residues which are connected via β-1,3-linkages. In every 40-50 glucose residues, the carbon-6 positions become sites for attachment of additional β-1,3-glucans to generate a branched structure (Manners et al., 1973; Fontaine et al., 2000). Branched glucans provide mechanical strength and integrity to the cell wall by cross-linking with each other, as well as with mannanproteins (Kollár et al., 1997).

1.1.4 Callose in plants

In contrast to extensive studies on cellulose, a similar linear polysaccharide that accounts for 15-30% of the dry mass of all primary cell walls, research on callose and the molecular mechanism of its synthesis in plants has begun relatively recently (Verma and Hong, 2001; Dong et al., 2005; 2008; Dhugga, 2012). Callose is synthesized in the cell walls of specialized cells during specific stages of development.
Even though callose is accumulated in the cell wall at a lower level compared to cellulose, it is biologically significant as it plays crucial roles in various biological processes during plant growth, development, and also plant defense in response to adverse environmental stimuli (Chen and Kim, 2009). Callose is required for cell plate formation during cell division and cytokinesis, and is an important part of vascular bundles, pollen wall exine, and trichomes (Píršelová and Matušíková, 2013). Callose deposition at plasmodesmata (PD) and its subsequent degradation are critical for the regulation of symplastic movement of various substances. It is also important for formation and closing of sieve plate pores. Thus, callose plays an important role in cell-to-cell communication, and is required for proper development at different stages (Levy et al., 2006; Lucas et al., 2009; Xu and Jackson, 2010; Maule et al., 2011; Zavaliev et al., 2011; Xie et al., 2012). Most of the recent knowledge regarding the function of callose in plant development has been generated through studying *Arabidopsis thaliana* knockout lines for genes encoding callose synthases (Píršelová and Matušíková, 2013).

### 1.1.5 Role of callose in cytokinesis and cell plate formation

Cytokinesis in plants is different from that in animals because of some unique properties which can be considered as a type of polarized secretion (Assaad, 2001). At the end of anaphase of cell division, the components required for cell wall formation are carried to the equator of the dividing cell by secretory vesicles that are derived from Golgi. Later, these vesicles will fuse to form a membrane-attached structure, called the tubulovesicular network, which will give rise to the cell plate (Söllner et al., 2002). The cell plate will keep expanding until it binds to the mother cell wall at the division site. Callose deposition at the tubulovesicular network enforces widening and consolidation of tubules leading to conversion of the network into a fenestrated sheet (Samuels et al., 1995) to prevent the swelling of this structure (Verma, 2001). Cell plate maturation will proceed by callose replacement with cellulose and pectin that will eventually form the cell wall (Kakimoto and Shibaoka, 1992; Samuels et al., 1995). Therefore, a delay or absence of callose biosynthesis, as well as its overproduction can cause alteration of the cell plate composition and generate daughter cells with different ploidy levels (Verma, 2001) (Fig. 1.2).
Figure 1.2 Formation of defective cell plate can lead to abnormal mitosis. (A) Abortion of cell division after formation of nuclear envelope causes packaging of two newly formed nuclei into one cell and generation of binucleated cell and, therefore, ectopic polyploidization. (B) If the cell division abortion occurs before nuclear envelope formation, even though the sister chromatids have already been separated, they will be encapsulated into the same nucleus and, therefore, cells with different ploidy levels, which is known as ectopic endomitosis, will be generated.
Defects in cytokinesis can induce ectopic endopolyploidy which has been reported for several organisms and cell types (Hatzfeld and Buttin, 1975; Thompson and Lindl, 1976; Karess et al., 1991; Castrillon and Wasserman, 1994; Neufeld and Rubin, 1994; Liu et al., 1997; Spielman et al., 1997; Lordier et al., 2008; Pampalona et al., 2012; Serres et al., 2012). Cell plate-destructive conditions can also mimic ectopic endopolyploidization (Röper and Röper, 1977; De Storme et al., 2012). The cytological mechanism responsible for nuclear fusion in cell-plate defective mutants, resulting in enlarged cells with either multi-nucleated or polyploid nuclei, is not yet clear.

1.1.6 Role of callose in plasmodesmata regulation

Unlike animal cells, plant cells have stiff cell walls and do not move after cell division. Therefore, determination of a plant cell identity is highly dependent on positional cues. Accordingly, exchange of information between cells is required. Intercellular signaling processes occur through either apoplastic signaling using ligands and transmembrane-localized receptors, or symplastic movement of molecules (Stahl and Simon, 2013). Plant cells are interconnected via narrow channels of PD that facilitate symplastic movement of nutrients and signaling molecules (Rinne and van der Schoot, 1998) (Figure 1.3).

The PD pores that are formed between cells, allowing macromolecular trafficking through the cell wall barrier, are more than fixed passive channels. Instead, they dynamically allow passing of molecules of different sizes and are regulated by the production and/or degradation of callose (Vatén et al., 2011; Zavaliev et al., 2011). It has been proposed that callose is accumulated in the apoplastic neck of the PD to regulate its connectivity (Kitagawa et al., 2015).

Reversible deposition of callose seems to be an important mechanism to control PD permeability and regulates the PD’s size exclusion limit (SEL) (Yadav et al., 2014). SEL determines the size of the PD pores and is typically defined based on the size of the largest molecule that can pass through PD aperture. The PD SEL is mostly shown by kDa as its unit (Kempers and van Bel, 1997; Oparka and Cruz, 2000). For example, in poplar and birch, PD in shoot apical meristems (SAMs) are closed during the dormancy
**Figure 1.3** Diagram of a simple primary PD.

Primary PD consist of a narrow cytoplasmic channel which extends across the cell wall with an appressed endoplasmic reticulum (ER) tubule passing through its middle connecting two neighboring cells. It has been suggested that callose deposition at the neck of the PD modulates the SEL of PD and its permeability. PD facilitate symplastic movement of nutrients and different signaling molecules including mobile transcription factors and miRNAs. Figure modified from Morgan et al. (2010).
dormancy period via the deposition of callose. However, after induction of dormancy release by chilling, open PD are restored through degradation of callose by activity of β-1,3-glucanases (Rinne et al., 2001; 2011a). Although genetic, molecular, and biochemical studies during the last decades have implicated the important role of callose equilibration at the PD in regulation of PD SEL and symplastic movement, the molecular mechanism(s), which can clearly link the identified players for endogenous signaling to callose homeostasis, is not well-known yet (De Storme and Geelen, 2014).

1.2 Callose biosynthesis

Uracil-diphosphate glucose (UDP-glucose) is the precursor for callose biosynthesis (Chen and Kim, 2009). Biochemical and molecular studies in several plant species have shown that callose is synthesized by a class of enzymes, termed callose/glucan synthases (Verma and Hong, 2001; Brownfield et al., 2007; Brownfield et al., 2008).

1.2.1 Glucan synthase in fungi

Synthesis of (1,3)-D-glucan polymer in fungi is catalyzed by UDP-glucose β(1,3)-D-glucan β(3)-D-glucosyltransferases (Douglas, 2001). These enzymes are known to form membrane-associated complexes that use UDP-glucose as a substrate and produce a linear polysaccharide. Several lines of evidence have suggested that the reaction is progressive. Chains of polysaccharide with increasing length are produced by adding one mole of glucose for hydrolyzing each mole of UDP-glucose (Shematek et al., 1980). Even though more than a hundred β-1,3-glucan synthase genes in fungi are available in public databases (Yang et al., 2014), only a few fungal genes encoding a β-1,3-glucan synthase have been characterized (Park et al., 2014). In *Saccharomyces cerevisiae*, three homologues of FKSs, which encode the enzymes forming a complex with β-1,3-glucan synthase activity, have been identified. FKS1 is active in the cell during the vegetative growth phase of yeast. β-1,3-glucan required during sporulation is synthesized by FKS2 (Douglas et al., 1994; Mazur et al., 1995), while FKS3 is needed for spore wall formation (Ishihara et al., 2007). In contrast to *S. cerevisiae*, only one homologous gene for *FKS1* (*fksA*) has been identified and isolated from *Aspergillus nidulans* (Kelly et al., 1996). The
subcellular localization of the β-1,3-glucan synthases and their regulatory interactors in filamentous fungi remain to be investigated.

1.2.2 Glucan synthase-like proteins in plants

Glucan synthases in Arabidopsis were first identified through their sequence similarity with the β-1,3-glucan synthase catalytic subunit FKS1 of yeast (Hong et al., 2001a). The homologs of glucan synthase in yeast, FKS1 and FKS2, are regulated by Rho1 GTPase that modifies their phosphorylation status. In plants, however, the activity of glucan synthases is usually dependent on Ca\(^{+2}\) (Qadota et al., 1996; Calonge et al., 2003).

The Arabidopsis genome has 12 genes encoding GLUCAN SYNTHASE-LIKE (GSL) (Richmond and Somerville, 2001), also called CALLOSE SYNTHASE (CALS) (Verma and Hong, 2001). GSL genes mostly have 40-50 exons except for GSL1 and GSL5 that only have two and three exons, respectively (Enns et al., 2005). GSLs code for large integral membrane proteins with multiple predicted transmembrane helices. The transmembrane domains are clustered into two regions (N-terminal and C-terminal), leaving a large hydrophilic cytoplasmic loop in between (Töller et al., 2008). The hydrophilic loop region possesses several glycosylation and phosphorylation sites. Therefore, it might act as an interaction and catalytic site for several regulatory proteins and substrates such as UDP-glucose (Verma and Hong, 2001). However, no specific domain containing a binding site for UDP-glucose has been identified yet (Brownfield et al., 2009; Zavaliev et al., 2011).

GSLs are known as enzymes synthesizing callose in response to different developmental, physiological, and environmental signals and in various tissues in plants (Verma and Hong, 2001). GSL isoforms play various roles during plant development. The actual organization of these enzymes, their stoichiometry and regulation are not well understood. Several studies have shown that a series of intracellular and extracellular factors and signals might be required to regulate GSLs’ enzyme activity (Hayashi et al., 1987; Andrawis et al., 1993; Li et al., 1997, 1999a; Cui et al., 2001).
In Arabidopsis, *GSL1* and *GSL5*, differ from the other 10 genes of the family with having fewer and shorter introns. *GSL1* and *GSL5* are both located on chromosome 4, being about 8 centimorgan (cM) apart from each other. They also show similar expression patterns in roots, rosette leaves, stems, and flowers (Enns et al., 2005). Both genes have been shown to be involved in sporophytic development in Arabidopsis, where *GSL5* plays a more substantial role. Studies indicate that *GSL1* and *GSL5* are required for callose formation in the cell wall that separates the tetraspores, but are not needed for callose deposition at the wall surrounding the pollen mother cells. Since *GSL2* is highly expressed in the pollen, it might be the one that is involved in callose biosynthesis in the wall of pollen tubes (Doblin et al., 2001; Becker et al., 2003).

*GSL2* was shown to be localized to the plasma membrane and Golgi-associated endomembrane structures (Xie et al., 2012) (Doblin et al., 2001; Becker et al., 2003). However, callose deposition was detected only in the cell wall and not in the Golgi vesicles. It is predicted that the plasma-membrane localized *GSL2* might be the ‘active’ form of the enzyme whereas the endomembrane-associated *GSL2* may be the ‘inactive’ form. It is still not clear what function the inactive form of the enzyme contributes in this intracellular compartment (Xie et al., 2012).

There are no studies available on *GSL3* and its role in plant development. According to microarray data (Schmid et al., 2005), *GSL3* is highly expressed during embryo development. At the amino acid sequence level, *GSL3* shows the highest similarity to *GSL6*.

*GSL4* mutations were shown to have no substantial impact on the plant phenotype or deposition of callose, indicating that *GSL4* enzyme activity does not play a major role during plant development (Maeda et al., 2014).

*GSL5* is the best characterized member of the GSLs family in Arabidopsis and is known as a callose synthase that is required for formation of callose in response to fungal pathogens (Wawrzynska et al., 2010; Ellinger et al., 2013; Kopischke et al., 2013; Ellinger et al., 2014). A *GSL5* knockdown line, generated by a double-stranded RNA interference (dsRNAi) approach, showed absence of papillary callose deposition after inoculation of leaves with the fungal pathogens. Although the typical round wall
appositions, which is formed beneath fungal appressoria, was indistinguishable from wild type, the cell walls lacked callose in the gsl5 knockdown lines (Jacobs et al., 2003). It has been suggested that GSL5 plays a role in stimulating the activity of pre-existing callose synthase enzyme, as well as specifically targeting the newly synthesized enzyme to the cellular “stress site” (Jacobs et al., 2003). It has been recently suggested that tocopherols, a family of vitamin E compounds, might be required for post-translational activation of GSL5 through a mechanism which is unlikely to be dependent on the wound-signaling pathway (Maeda et al., 2014).

GSL6 is a cell plate-specific callose synthase localized at the cell plate of cells during cytokinesis and has been suggested to form a complex with a UDP-glucose transferase (Hong et al., 2001a; Hong et al., 2001b). GSL6 expression is up-regulated in response to salicylic acid (SA), indicating that, similar to GSL5, it might be involved in callose deposition after pathogen attack. gsl6 mutant plants do not show any detectable phenotypic defects under regular growth conditions (Dong et al., 2008).

GSL7 is only expressed in the vascular system and more specifically in the phloem sieve elements and companion cells, indicating that GSL7-mediated callose synthesis is highly tissue-specific (Xie et al., 2011). In support of this, gsl7 mutants do not display any obvious macroscopic phenotypic defects, suggesting that GSL7 has no biological function other than phloem-specific PD callose synthesis (Huang et al., 2009). GSL7 is highly and specifically expressed in phloem during root development, when a large number of symplastic connections are required (Vatén et al., 2011).

According to a promoter analysis (Dong et al., 2008) and microarray data (Schmid et al., 2005), GSL9 is poorly expressed in most of the tissues and organs compared to the other GSLs. At the amino acid sequence level, GSL9 is most closely related to GSL12, but there is no evidence available yet to show their functional redundancy.

Although among all members of the GSL family, GSL10 is most closely related to GSL8, with 63.7% DNA and 63.4% protein sequence identity, respectively, it acts independently from GSL8 in different sporophytic tissues and organs, and during plant growth (Zimmermann et al., 2004; Töller et al., 2008). It has been shown that GSL8 and GSL10
are independently required during pollen development. In gsl10 mutants, entry of microspores into mitosis is prevented (Töller et al., 2008), therefore, efforts to obtain homozygous knockouts have not been successful.

Similar to gsl4, gsl6 and gsl7, gsl11 mutant plants do not show any noticeable growth defects compared to wild-type plants. Loss of enzyme activity does not reduce callose deposition (Maeda et al., 2014), suggesting that GSL11 does not play a major role and probably is not required for plant development. The possibility of having a more specialized role cannot be ruled out.

GSL12 synthesizes callose at certain sites in the cell walls surrounding the PD and, therefore, can determine PD size and control molecular trafficking through PD. During plant development, GSL12 is highly expressed in the phloem, stele and root meristem (Vatén et al., 2011). Expression of GSL12 in a gsl7 background can complement the mutant phenotype (Xie et al., 2011). GSL7 is responsible and required for callose biosynthesis in the phloem and during phloem development (Barratt et al., 2011). These observations led to the proposal that these two proteins are, at least partially, if not completely, redundant regarding their function during sieve elements development, (Vatén et al., 2011).

1.2.3 GLUCAN SYTHASE-LIKE 8

GLUCAN SYTHASE-LIKE 8 (GSL8) is known as one of the few members of the GSL family with ubiquitous expression in most of the tissues and organs during plant development with the highest expression in vasculature (Schmid et al., 2005). Since most of the callose in the vasculature is deposited at the PD to reduce SEL of PD, GSL8 has been suggested to be associated with PD regulation. According to genomic and amino acid sequence, GSL8 is most closely related to GSL10, and is also required during pollen development with an important role in sporophyte (Töller et al., 2008).

GSL8 callose biosynthesis activity is required for proper cell plate formation during cytokinesis. massue, a mutant allele of GSL8, showed significant delay in cell plate formation, similar to that reported for cytokinesis defective mutants such as knolle and
"keule" (Thiele et al., 2009). Although a direct link between callose deposition at the cell plate and cytokinesis defects was not confirmed, the incomplete cell walls and multinucleated cells in massue were explained as downstream effects of defects in callose deposition (Thiele et al., 2009). A substantial role of GSL8 in proper cell plate constitution during cytokinesis was further confirmed using an inducible RNA interference (RNAi) line of GSL8 (Chen et al., 2009). gsl8 mutant seedlings showed stubs in their cell walls, two nuclei in one cell, excessive cell proliferation at the epidermis, and clusters of stomata (Chen et al., 2009). A weak mutant allele of GSL8, called enlarged tetrad2 (et2), showed induction of endomitotic polyploidization in different tissues due to defects in cytokinesis and cell wall establishment, indicating that callose is required for retaining the ploidy level. Different from other gsl8 mutant alleles, in et2 plants cytokinesis impairments do not induce serious damage on the plant’s vegetative growth and its normal development, therefore, et2 plants appear similar to the wild type (De Storme et al., 2013). Even though several indirect lines of evidence have already confirmed callose requirement for cell plate growth and cytokinesis, no direct functional evidence is yet available.

Guseman et al. (2010) showed that CHORUS, another allele of GSL8, is required for formation of normal stomatal patterns and organizing stomatal cell lineage initiation. The numbers of stomatal-lineage cells were significantly increased in chorus mutant plants, and this increase was mediated by SPEECHLESS (SPCH) (Guseman et al., 2010), a basic helix-loop-helix (bHLH) protein that specifically initiates stomatal lineage (Lampard et al., 2008). In chorus, there was also an increase in symplastic movement of macromolecules between epidermal cells, and larger molecules which would normally not pass through the PD could diffuse to the neighboring cells, suggesting that in addition to cytokinesis defects, gsl8 mutants exhibit PD defects as well (Guseman et al., 2010). The gsl8 mutant’s pleiotropic phenotype suggests that GSL8 might have important regulatory roles during plant development, rather than just being an enzyme responsible for callose biosynthesis at the cell plate to complete cytokinesis and at the PD to regulate their SEL.
1.2.4 Callose synthase complex

Highly regulated synthesis and deposition of callose indicates that an extremely-specialized protein complex is involved in this process (De Storme and Geelen, 2014). It is predicted that GSLs might be integrated into a complex, hypothetically named the CalS complex. Based on the genetic evidence obtained from studying de novo cell plate formation, pollen tube tip growth and cotton fiber elongation, there are at least four proteins which can potentially comprise the CalS complex, including at least one of the GSLs, UDP glucose transferase1 (UGT1), Rho-like GTPase (ROP), and sucrose synthase (SuSy) (Andrawis et al., 1993; Amor et al., 1995; Shin and Brown, 1999; Hong et al., 2001a; 2001b; Verma and Hong, 2001). Degradation of sucrose is catalyzed by UDP-glucose:D-fructose 2-alpha-D-glucosyltransferase (SuSy), a sugar metabolic enzyme which has been characterized as a critical part of cellulose synthase complexes to provide UDP-Glc. Since GSLs also use UDP-Glc as the precursor for β-1,3-glucan polymer synthesis, it is tempting to speculate that SuSy might provide the supply of UDP-Glc to GSLs as well. Supporting this hypothesis, UDP-Glc transfer from SuSy to GSLs occurs in presence of UGT1 activity (Hong et al., 2001a; 2001b). UGT1 interacts with Rho-like GTPase 1 (ROP1) and this interaction only takes place in GTP-bound state of UGT1, indicating that UGT1-dependent activity of ROP1 might regulate GSLs by restricting their substrate supply (Li et al., 1999b; Verma and Hong, 2001). UGT1, ROP1 and SuSy lack transmembrane domains. Therefore, if there is any sort of interactions between these proteins and GSLs, it should be through their association with the hydrophilic loop of GSLs (De Storme and Geelen, 2014).

Although the significance of a CalS complex and its components required for callose deposition at the cell plate during cytokinesis has been well studied and characterized, it is not clear if all of these subunits are needed for synthesizing callose at the PD. It has been suggested that CalS complex composition likely differs depending on the tissue and process that it is involved in (Verma and Hong, 2001). Hence further research is required to reveal if the CalS complex at the PD is closely-related to the one at the cell plate.
1.3 Regulatory role of plasmodesmata in development

During plant development and in response to biotic and abiotic stresses, cell walls undergo structural modifications that are predicted to introduce changes in PD structure (Knox and Benitez-Alfonso, 2014). Genetic screening to identify mutants defective in intercellular communications has confirmed that PD components are required for viability as functional or structural PD mutants are mostly lethal (Wu et al., 2002). The number and SEL of PD vary depending on the tissue and developmental state (Wu et al., 2002). SEL restricts passage of those molecules with sizes larger than SEL through PD (Crawford and Zambryski, 2000).

Cell wall PD can be primary or secondary. Primary PD are formed during cell division when the cell plate is forming, while secondary PD are formed in localized breaks in the established cell walls. Both primary and secondary PD contain a membranous channel and a desmotubule that is connected to the ER of both cells (Fig. 1.3) (Burch-Smith and Zambryski, 2012). PD plasma membrane has a distinct lipid composition compared to the other regions. It consists of complex sphingolipids, sterols and glycerolipids, suggesting that they may have a role in signaling for a variety of receptor proteins, some of which have shown specialized localization and/or activity at either PD or plasma membrane (Kitagawa et al., 2015).

While PD were previously considered to be channels used for passive movement of water, nutrients, and ions based on their concentration gradients, newer research has shown PD’s function is not restricted to passive diffusion. It is now well known that movement of larger molecules is also facilitated by PD and is more selective than a gradient-based movement (Burch-Smith and Zambryski, 2012). Transcription factors, metabolites, and small RNAs, which are responsible for cell-to-cell communication during plant development and for plant defense upon virus infection, are transported through PD (Stahl and Simon, 2013).

PD are required for symplastically restricted movement of plant virus pathogens and their invasion to neighboring cells (Lucas, 2006). Virus transport upon infection is affected by formation of complex PD and callose biosynthesis at the PD which are regulated by SA
A range of molecular interactions between host and pathogen are required by viruses to access PD. Two models have been suggested to describe the movement of viruses via PD. In the first model, viral replication complexes (VRCs) can be translocated, i.e. Tobacco mosaic virus (TMV), in which VRCs are formed intracellularly via its interaction with ER-microtubule junctions at PD (Heinlein, 2015). In the second model, encapsulated viral RNA movement plays an important role. Potato virus X (PVX) cap proteins have been detected on both sides of PD, suggesting that viral replication occurs at PD and this seems to be required for intercellular movement of PVX (Tilsner et al., 2014). Biotrophic fungi invasion into plant tissues has also been suggested to be facilitated by PD (Kankanala et al., 2007). All of these macromolecules and organisms have sizes above the normal SEL of PD, indicating the importance of PD regulation to recognize and transport highly specific molecules between cells (Thomas et al., 2008).

1.4 Protein constituents of plasmodesmata

Secondary PD are formed de novo (Faulkner et al., 2008). Twined, Y- and H-shaped (branched) PD are probably the intermediates during the process of PD de novo formation (Maule, 2008). PD are known as complex structures. Attempts to purify PD have not been successful. Therefore, their molecular composition and the mechanisms that regulate them are not well understood. Since cell-to-cell communication is required for survival, PD mutants exhibit severe developmental defects as early as embryogenesis (Burch-Smith and Zambryski, 2012). In recent years, by taking advantage of improved methods, attempts towards isolating cell wall fractions harboring PD have become more successful. More than a thousand PD-associated candidate proteins have been identified using nano-liquid chromatography and an Orbitrap ion-trap tandem mass spectrometer in combination with using Arabidopsis cell cultures as plant material (Fernandez-Calvino et al., 2011).

Distinguishing PD proteins from other plasma membrane proteins has remained a challenge due to lack of a specific motif/domain. PD proteome analyses revealed association of several cell wall remodeling enzymes, as expected (Table 1.1). This set of enzymes is involved in cell wall modification and remodeling primarily with a role in
Table 1.1 List of a subset of proteins previously identified in the PD proteome.
The identified proteins have putative functions important for cell wall modifications and remodeling (Brecknock et al., 2011; Fernandez-Calvino et al., 2011).

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<td>AT5G42100</td>
<td>Callose metabolism</td>
</tr>
<tr>
<td>β-1,3-GLUCANASE</td>
<td>AT5G58090</td>
<td>Callose metabolism</td>
</tr>
<tr>
<td>PECTIN METHYLESTERASE 1</td>
<td>AT1G53840</td>
<td>Pectin modification</td>
</tr>
<tr>
<td>PECTIN METHYLESTERASE 26</td>
<td>AT3G14300</td>
<td>Pectin modification</td>
</tr>
<tr>
<td>PECTIN METHYLESTERASE INHIBITOR 1</td>
<td>AT4G12390</td>
<td>Pectin modification</td>
</tr>
<tr>
<td>POLYGALACTURONASE/PECTINASE</td>
<td>AT1G65570</td>
<td>Pectin modification</td>
</tr>
<tr>
<td>GLYCOSYL TRANSFERASE ACTIVITY</td>
<td>AT1G02730</td>
<td>Biosynthesis of cell wall</td>
</tr>
<tr>
<td>UDP-GLYCOSYL TRANSFERASE</td>
<td>AT3G46650</td>
<td>Biosynthesis of cell wall</td>
</tr>
<tr>
<td>PLASMODESMATA-LOCATED PROTEIN 1</td>
<td>AT5G43980</td>
<td>Plasmodesmal structural protein</td>
</tr>
<tr>
<td>PLASMODESMATA-LOCATED PROTEIN 6</td>
<td>AT2G01660</td>
<td>Plasmodesmal structural protein</td>
</tr>
<tr>
<td>ACTIN 1</td>
<td>AT2G37620</td>
<td>Structural constituent of cytoskeleton</td>
</tr>
<tr>
<td>ACTIN 7</td>
<td>AT5G09810</td>
<td>Structural constituent of cytoskeleton</td>
</tr>
<tr>
<td>ACTIN 8</td>
<td>AT1G49240</td>
<td>Structural constituent of cytoskeleton</td>
</tr>
<tr>
<td>MYOSIN VIII A</td>
<td>AT1G50360</td>
<td>Glucuronoxylan metabolism</td>
</tr>
<tr>
<td>MYOSIN XI K</td>
<td>AT5G20490</td>
<td>Actin filament-based movement</td>
</tr>
</tbody>
</table>
callose biogenesis and homeostasis, including glucan synthase-likes (GSLs) and β-1,3-glucanases (BGs) (Gaudioso-Pedraza and Benitez-Alfonso, 2014). Even though pectin metabolism-related proteins have been detected in PD proteome, there is not enough information on how these enzymes might be involved in regulating symplastic intercellular communication. Pectin seems to be important for stabilizing PD anchorage to cell walls. Therefore, lack of pectin can presumably destabilize PD channel structure (Yu et al., 2004).

PD proteome revealed other enzymes involved in general cell wall biosynthesis and assembly, including glycosyl transferases and reversibly glycosylated polypeptides (RGPs). It is not known yet how expression of these proteins can modify PD structure and function (Pagant et al., 2002; Kong et al., 2012). An independent study revealed binding of RGPs class 1 family members to PD (Sagi et al., 2005). Reversible glycosylation of these proteins with UDP-sugars makes them a potential substrate for the enzymes involved in the biosynthesis of cell wall components. Overexpression of these proteins can increase callose accumulation which consequently restricts virus spreading and photoassimilates transport to sink tissues (Zavaliev et al., 2010; Burch-Smith et al., 2012). A novel regulator of symplastic trafficking was identified during a genetic screen for stomata differentiation mutants and was named KOBITO 1 (KOB1) (Kong et al., 2012). KOB1 encodes a glycosyl transferase-like protein and is involved in cellulose biosynthesis (Pagant et al., 2002). However, it is not clear how cellulose biosynthesis and PD permeability might be linked (Kong et al., 2012). It has been hypothesized that KOB1 might regulate cell-to-cell connectivity at the PD by providing carbohydrates supply for the CalS complex. To address this hypothesis, subcellular localization of KOB1 and its interaction with other protein constituents of PD should be further investigated (De Storme and Geelen, 2014).

The identity of the proteins detected in the PD proteome analyses, mostly with roles in biosynthesis and modification of cell wall components, needs to be further studied. Independent modification of these cell wall microdomains can potentially provide more detailed information on their specific role in regulating PD aperture and its SEL (Knox and Benitez-Alfonso, 2014).
1.4.1 Plasmodesmata-localized proteins

PLASMODESMATA-LOCALIZED PROTEIN 1 (PDLP1) to PDLP8 belong to a small subfamily of the RECEPTOR-LIKE PROTEINs (RLPs) family with two conserved Cys-rich repeats and two extracellular DUF26 domains. PDLPs have a distinct N-terminal domain that is exposed to the apoplast and can act as a receptor for extracellular signals (Thomas et al., 2008). Since PDLPs have extremely short cytoplasmic domains (less than 20 amino acids) with no known signaling capacity, it has been proposed that the extracellular domains of PDLPs are their interacting domains with other proteins. PDLPs’ interaction with other modifying enzymes, which are localized at the PD, can theoretically initiate signal transduction (Thomas et al., 2008). PDLP1-overexpression lines exhibit a reduction in GFP cell-to-cell diffusion, suggesting that PDLP1 is a negative regulator of trafficking through PD (Thomas et al., 2008). None of the PDLPs single knockouts show either phenotypic defects or changes in symplastic movement, which suggests that members of the PDLP family are highly redundant (De Storme and Geelen, 2014). Thomas et al. (2008) demonstrated that PDLPs’ transmembrane domain (TMD) is required and sufficient for their localization to the PD, while the C-terminal cytoplasmic domain is less likely to be required for their intercellular targeting. The receptor-like targeting of PDLPs and their localization at the PD suggest that extracellular signaling can also have a role in controlling symplastic trafficking (De Storme and Geelen, 2014). Therefore, PDLPs might play a role during plant development, and in response to both internal and external signals (Lee et al., 2011).

PDLPs might negatively regulate PD SEL by inducing callose deposition at the PD, which might be mediated through their interaction with callose homeostasis proteins such as GSLs (De Storme and Geelen, 2014). Key evidence supporting this hypothesis was obtained from PDLP5-overexpression plants, in which an increase in callose deposition at the PD and concomitant reduction in cell-to-cell movement of viral genome movement proteins (MPs) was observed. This finding suggested that PDLP5 controls PD size exclusion limit through modification of callose accumulation at the PD in response to pathogen invasion, also confirmed by strong up-regulation of PDLP5 upon bacterial infection (Lee et al., 2008; 2011). A recent study has shown that callose accumulation at
PD is mediated by PDLP5 and is dependent on both the presence and an increase in SA. This finding suggested that both PDLP5 and SA are required and are likely to act interdependently to reduce cell-to-cell permeability and symplastic movement (Wang et al., 2013).

Interestingly, contrary to PDLPs’ negative regulation of PD, under specific conditions PDLPs can also positively regulate cell-to-cell communication through PD. It has been recently reported that the MPs from tubule-forming *Grapevine fan leaf virus* (GFLV) can physically interact with all PDLPs isoforms (Amari et al., 2010). Additionally, MPs targeting to the PD, MP tubule assembly and GFLV PD-mediated movement require this interaction with PDLPs (Amari et al., 2010). Hence, further research is required to identify the molecular mechanisms and factors underlying these two different functions of PDLPs: induction of callose deposition in response to pathogens, and facilitation of the viral genome spreading.

### 1.4.2 β-1,3-Glucanases

β-1,3-glucanases or glucan endo-1,3-β-glucosidases (BGs) are enzymes that hydrolyze the linkages in 1,3-β-D-glucosidic polymers and generate single β-1,3-glucan subunits. These enzymes are not restricted to plant species and can also be found in bacteria, fungi, metazoa and viruses (Bachman and McClay, 1996; Sun et al., 2000).

BGs were first identified due to their role in protecting plants against pathogens (Kauffmann et al., 1987; Bowles, 1990; Sela-Buurlage et al., 1993; Stintzi et al., 1993; Jach et al., 1995; Douglas, 2001). BGs in Arabidopsis are a family with 50 members (Levy et al., 2007), and belong to glycosylphosphatidylinositol-anchored proteins (GPI-APs) (Elortza et al., 2003). GPI-APs have a specific plasma membrane localization pattern known for the absence of TMDs. BGs bear a cleavable hydrophobic N-terminal secretion signal peptide for translocation into the ER, and a hydrophobic C-terminal tail, which most likely forms a transient TMD, for targeting them to PM (Elortza et al., 2003). Three members of the BG family, ATBG_PPAP, PdBG1, and PdBG2, were shown to have a role in callose degradation at the PD (Benitez-Alfonso et al., 2013).
Genetic characterization of PD-localized enzymes which degrade callose suggests their requirement for balancing callose level at the PD. In support of this hypothesis, atbg_ppap mutants show an increase in callose accumulation at the PD as well as decrease in the systematic spread of the TMV and Turnip vein clearing virus (TVCV) (Levy et al., 2007; Zavaliev et al., 2013).

PdBG1 and PdBG2 localization at the PD was confirmed by their punctate distribution pattern at the cell periphery (Benitez-Alfonso et al., 2013). Although single mutants do not exhibit any phenotypic defects, most likely due to functional redundancy, double mutants (pdbg1 pdbg2), show an increase in callose accumulation at the PD as well as restricted cell-to-cell transport of macromolecules. PdBG1-overexpression plants show the opposite effect. Therefore, it can be concluded that callose accumulation at the PD and symplastic movement in the developing roots are negatively regulated by two redundant β-1,3-glucanases (PdBG1 and PdBG2) (Benitez-Alfonso et al., 2013).

Involvement of β-1,3-glucanases in PD regulation has also been observed in other plants. For example, in Populus, two closely-related genes encoding PD-located BGs were identified with roles in mediating FLOWERING LOCUS T (FT) movement into the bud during the dormancy release period (Rinne et al., 2011b). BGs’ involvement in intercellular trafficking regulation has been identified mostly in permanent processes, suggesting that callose turnover mechanisms are used by plants for longer-lasting closure of PD (Levy et al., 2007). It needs to be further investigated whether plants employ other mechanisms for short-term changes in the PD channels.

1.5 Symplastic movement of mobile factors through plasmodesmata

PD connect almost all plant cells providing selective passage of macromolecules (Crawford and Zambryski, 2000; Lucas and Lee, 2004). There are a number of transcription factors, in shoot and root apical meristems (SAMs and RAMs, respectively), which are expressed in different cells from where they are localized and have functions, known as non-cell-autonomous transcription factors (Kurata et al., 2005a; Lee et al., 2006). Transcription factors that are involved in development, particularly KNOTTED 1-LIKE HOMEBOX (KNOX) and MCM1, AGAMOUS, DEFICIENS and SRF (MADS)
family members, exhibit intercellular movement, potentially through PD (Xu and Jackson, 2010; Burch-Smith et al., 2011). Similar to transcription factors, some small RNAs, particularly those playing roles in RNA silencing pathways, also show non-cell-autonomous activity (Dunoyer et al., 2005).

1.5.1 Mobile transcription factors

Plants, similar to animals, use signal peptides, hormones, and microRNAs (miRNAs) for intercellular signaling. They are, however, additionally equipped with a collection of mobile transcription factors to deliver intercellular signals (Han et al., 2014a). Since plant cells are surrounded by rigid cell walls, the movement of most of the mobile proteins is facilitated via PD, which provides a direct route for protein movement between cells (Oparka, 2004).

Co-operation and co-ordination of individual cells is required for the development of multicellular organisms. Intercellular communication between cells is mandatory to co-ordinate proliferation, differentiation, and programmed cell death during tissue formation. Precise control of intercellular signaling during development is the key to generate a multicellular organism (Long et al., 2015). Movement of molecular signals plays important roles in many highly regulated cell proliferation and specification phenomena. However, the molecular context of all these signals is not yet entirely known. Regulation of trichome and root-hair patterning (Bouyer et al., 2008; Digiuni et al., 2008; Pesch and Hülskamp, 2009; Wester et al., 2009), RAM and SAM development (Lucas et al., 1995; Kim et al., 2003; Schlereth et al., 2010; Xu et al., 2011), and the patterning and development of the mature root (Nakajima et al., 2001; Gallagher et al., 2004; Kurata et al., 2005b; Cui et al., 2007) are all mediated by mobile transcription factors.

Root tissue patterning is determined by a group of pluripotent cells in the meristematic tissue surrounding the quiescent center (QC) cells (Dolan et al., 1993; Van den Berg et al., 1997). Communication between these cells and those around them is achieved by different mobile signals, including the phytohormone auxin and mobile proteins or peptides (Sabatini et al., 1999; Stahl et al., 2009; Matsuzaki et al., 2010; Schlereth et al.,
2010). Although the concept of mobile transcription factor signaling seems to be simple, several pieces of evidence have revealed an astonishing complexity of this process.

Since KNOTTED 1 (KN1), a homeodomain protein from Zea mays, was characterized as the first mobile transcription factor (Hake and Freeling, 1986; Lucas et al., 1995), the list of mobile transcription factors, also called non-cell-autonomous transcription factors (NCATFs), has been growing continuously. Some of the proteins with confirmed regulatory roles in plant development include LEAFY (LFY), a regulator of flower development (Sessions et al., 2000); SHORT-ROOT (SHR), a GRAS-domain transcription factor regulating stem cell activity and radial patterning of the Arabidopsis root (Nakajima et al., 2001); FT, a long-distance signal inducing floral transition (Corbesier et al., 2007); WUSCHEL (WUS), a homeodomain transcription factor maintaining stem cell homeostasis in the SAM (Yadav et al., 2011); PLETHORA 2 (PLT2), an APETALA 2 (AP2) transcription factor driving auxin-dependent root zonation (Mähönen et al., 2014); and many more (Han et al., 2014a).

Depending on the type of NCATF, the movement pattern can be different. If the protein size is smaller than the SEL of plasmodesmata, like LFY, the movement takes place via diffusion between plant cells similar to what happens with free green fluorescent protein (GFP) (Wu et al., 2003). However, cell-to-cell movement of most NCATFs is highly controlled, and is tightly linked to their activities and role in development.

1.5.2 SHORT ROOT

Intercellular movement of endogenously encoded SHR is essential for a plant’s normal growth and development (Nakajima et al., 2001; Gallagher and Benfey, 2009; Yadav et al., 2011). SHR is required for cell division, endodermis specification (Benfey et al., 1993; Helariutta et al., 2000) and turning on microRNA165/6 (miR165/6) in the endodermis (Carlsbecker et al., 2010). shr mutants show reduced root length, loss of a ground tissue layer and endodermal cell fate (Benfey et al., 1993; Scheres et al., 1995; Helariutta et al., 2000).
SHR protein is a transcription factor which has the ability to move from the stele cells, its domain of transcription, to a single layer of adjacent cells including QC, the cortex/endodermis initial (CEI) and daughter cells (CEIDs), as well as to all endodermis cells (Fig. 1.4) (Nakajima et al., 2001; Wu and Gallagher, 2013). SHR movement acts both as a signal from the stele and an activator of endodermal cell identity determination and cell division through the transcriptional activation of SCARECROW (SCR) (Nakajima et al., 2001). Ectopic expression of SHR in adjacent layers induces an increased number of cell layers and cell identity change (Cui et al., 2007). The protein’s subcellular localization differs depending on the tissue. In the stele cells, SHR is localized in both the nucleus and cytoplasm, while in the neighboring cell layer, including the QC, CEI, and endodermis, it specifically resides in the nucleus (Nakajima et al., 2001). SHR movement needs to be carefully regulated to guarantee both up- and down-regulation of asymmetric cell divisions in the endodermis (Vatén et al., 2011).

SHR moves through PD (Vatén et al., 2011). This movement is mediated through SHR interaction with the SHR binding protein, SHR-INTERACTING EMBRYONIC LETHAL (SIEL) (Gallagher et al., 2004; Gallagher and Benfey, 2009; Koizumi et al., 2011). Recent study showed that the movement of SHR is facilitated by endosomes (Wu and Gallagher, 2014). SHR can be localized to early and late endosomes. However, it is not clear how this localization can promote SHR movement. Two models have been proposed to explain endosome-mediated movement of SHR. In the first model, endosomes can interact with SIEL to facilitate SHR movement towards the plasma membrane and then diffuses to PD. In the second model, endosomes act as a platform where SHR can associate with SIEL as well as other proteins that can directly or indirectly facilitate its movement between cells (Wu and Gallagher, 2014).

### 1.5.3 WUSCHEL

Intercellular communication between different cell types within plants’ stem cell niches is required for both stem cells maintenance and their differentiation (Spradling et al., 2008; Rieu and Laux, 2009). Several studies have shown that WUSCHEL (WUS), a homeodomain transcription factor produced in the organizing center cells of SAM, is required and sufficient for stem cell specification (Laux et al., 1996). WUS transcription
**Figure 1.4** Diagram of SHR and miR165/6 intercellular movement in Arabidopsis root. After expression in the stele cells, SHR moves from stele into the neighboring cells including endodermis where it transcriptionally activates *SCR*. SHR-SCR complex activates miR165/6 in the endodermis. After activation, the mature miR165/6 move from endodermis to stele as well as from endodermis to cortex. miR165/6 suppress the family of *HD-ZIP III* genes in stele by targeting their transcripts.
is restricted to cells of the SAM organizing center, which is known to be critical for keeping the number of stem cells constant (Yadav et al., 2011). The CLAVATA (CLV) signaling pathway mediates this process (Fletcher et al., 1999; Brand et al., 2002). WUS can move laterally in the SAM for at least two cell layers (Yadav et al., 2011).

WUS is synthesized in the organizing center cells of the SAM from which it can then move to adjacent cell layers. Studies have shown that interference with WUS movement does not hinder its biological activity, but inhibits SAM regular function (Yadav et al., 2011). WUS protein directly activates CLV3 transcription through binding to its promoter after migrating to surrounding cells of SAM central zone. Misexpression of WUS in adjacent cells of SAM central zone (CZ) causes expansion of the stem cells and meristem proliferation (Brand et al., 2002; Yadav et al., 2010). Cell-to-cell communication via PD in the CZ of the SAM is required for stem cell activity. Analysis of endogenous WUS has shown that WUS trafficking through PD is more possible than via either secretion or endocytosis. Since WUS degradation in SAM stem cells causes meristem termination, it can be concluded that WUS might be one of the critical signals derived from the stem cell niche (Daum et al., 2014). However, more studies are required to characterize WUS function and its molecular mechanisms in regulating cell division and differentiation of stem cells, which are crucial for stem cell maintenance.

1.5.4 Movement of small RNAs through plasmodesmata

It has been shown that in plants and animals small RNAs, including miRNAs, small interfering RNAs (siRNAs) and trans-acting siRNAs (tasiRNAs) have the potential to move between cells and mediate cell-to-cell communication. Although several pieces of evidence have indicated the movement of siRNAs, tasiRNAs and miRNAs, the mechanism(s) of this movement remains to be uncovered (Juarez et al., 2004; Dunoyer et al., 2007; Lin et al., 2008; Tretter et al., 2008; Chitwood et al., 2009; Nogueira et al., 2009). miR165/6, two very closely related miRNAs that differ by only one single nucleotide in their sequence, regulate plant development by targeting the family of class III homeodomain leucine zipper (HD-ZIP III) genes including PHABULOSA (PHB) (Jung and Park, 2007; Zhou et al., 2007; Liu et al., 2009). It has been reported that the
transcription of *HD-ZIP III* family genes is mostly restricted to the vascular cylinder (Lee et al., 2006) whereas miR165/6 is produced in the endodermis and targets *HD-ZIP III* mRNA in the stele (Fig. 1.4) (McConnell et al., 2001; Emery et al., 2003; Prigge et al., 2005). Hence, a mobile signal should exist to assist miR165/6 to accomplish its goal. Several studies have shown that miR165/6 movement plays critical roles in both shoot apical meristem and in root patterning (Juarez et al., 2004; Chitwood et al., 2009; Nogueira et al., 2009; Carlsbecker et al., 2010).

miR394 was identified as another mobile miRNA (Knauer et al., 2013). miR394 is produced in the protoderm and represses the transcript encoding the F box protein LEAF CURLING RESPONSIVENESS (LCR) by moving to the distal meristem. This repression is critical for maintaining stem cell pluripotency by synergistically increasing the signaling from beneath the stem cell generated by WUS. miR394, similar to WUS, can move for a distance of about three cell layers (L1-L3) in the SAM (Knauer et al., 2013).

### 1.6 Regulation of plasmodesmata size exclusion limit

Although the detailed process of SEL regulation is still under debate, several lines of evidence support callose turnover as an important mechanism for regulation of PD and symplastic trafficking through it (Levy et al., 2007; Vatén et al., 2011; Sevilem et al., 2013). Deposition of callose at the PD can physically constrict the channel. Therefore, reducing the SEL or even blocking the PD results in blockage of symplastic trafficking (Sevilem et al., 2013; Kitagawa et al., 2015). The SEL of the PD cytosolic space is decreased through callose deposition in the neck of the PD channel. On the other hand, callose removal from the PD increases PD SEL and hence, allows larger molecules to either passively or actively traffic via PD. It has been suggested that the amount of callose accumulated at the PD is regulated by a balance between the activity of enzymes synthesizing callose (GSLs) and those degrading the β-1,3-glucan polymer (BGs) (Fig. 1.5) (Benitez-Alfonso et al., 2009; Chen and Kim, 2009; Vatén et al., 2011; Zavaliev et al., 2011).
Figure 1.5 Callose deposition at the neck region regulates PD permeability.
Proper regulation of PD is achieved through a balance between the activity of the enzymes synthesizing callose and the ones degrading it, called β-1,3-glucanases. Callose turnover at the PD determines the status of the PD to be open (left) or closed (right), resulting in free or blocked symplastic movement between cells, respectively. Figure modified from Maule et al. (2012).
1.7 Dynamic modulation of plasmodesmata connectivity

Given that PDs are membranous structures facing the cytoplasm, cell wall, and apoplast, their function and development can be modified under the effect of any cytoplasmic and/or apoplastic change (Knox and Benitez-Alfonso, 2014). Studies on protein composition of PD have shown that these membrane-embedded structures are enriched in receptor and receptor-like proteins with roles in signaling pathways that are important for regulation of cell-to-cell communication (Jo et al., 2011; Faulkner, 2013; Faulkner et al., 2013; Stahl et al., 2013). These receptors, such as PDLP5 (see section 1.4.1), receive the signals from environmental changes or cell wall composition at the PD, and will respond to them by adjusting PD SEL.

Recent studies have shown that reactive oxygen species (ROS) can modulate plasmodesmal structure through affecting callose accumulation at PD (Benitez-Alfonso et al., 2009; Zavaliev et al., 2011). An earlier study had also proposed that the apoplastic ROS may act as an agent for loosening cell walls, which can then physically affect PD formation and function (Gapper and Dolan, 2006). The dependence of PD structure on ROS was further confirmed in gfp arrested trafficking (gat) mutant. The gat mutants exhibit reduced transport of GFP from the phloem to surrounding tissues and hyperaccumulation of ROS in their root meristems with approximately doubled level of callose at PD (Benitez-Alfonso et al., 2009). GAT1 encodes a type m thioredoxin (TRX-m3) expressed in plastids of meristems and organ primordia.

Another molecule that has been shown to regulate PD formation and callose biosynthesis through interaction with redox signaling pathway is SA (Wang et al., 2013). Studies indicate that SA regulates PD indirectly via induction of PDLP5 expression. This induction will positively affect callose metabolic enzyme activity and, therefore, can regulate symplastic movement and response to pathogens (Fitzgibbon et al., 2013; Wang et al., 2013).

Interestingly, several reports in recent years have implicated phytohormones as another factor influencing symplastic communication. For example, auxin has been shown to be related to cell wall and PD changes. During the phototropic response, callose plays a role
in auxin gradient formation. Auxin can positively regulate \textit{GSL8} expression through a pathway in which the auxin response factor 7 (ARF7) is involved (Han et al., 2014b). Symplastic transport can be regulated by auxin through targeting several other callose metabolic genes. The expression of \textit{PLASMODESMATA CALLOSE-BINDING PROTEIN 1} (\textit{PDCB1}) is up-regulated in lateral root after auxin treatment (Maule et al., 2013). \textit{PDCB1} is also involved in callose deposition at the PD (Simpson et al., 2009). During later stages of lateral root emergence, \textit{PDCB1} function is required for callose deposition around the primordium to isolate it from the overlying tissue (Maule et al., 2013). It has been proposed that auxin-imposed modification of the PD might be through changes in pectin structure or distribution. An early study reported that auxin can induce pectin methylesterase (PME) activity (Bryan and Newcomb, 1954). PME, which was previously identified in the PD proteome (Table 1.1), is known as an enzyme that demethylesterifies homogalacturonan (HG) in the cell wall (Mohnen, 2008). Therefore, it is plausible that auxin affects cell wall-related mechanisms by interfering with pectin esterification, and ultimately causes alterations in PD transport (Knox and Benitez-Alfonso, 2014).

Further evidence supports the potential role of other small molecules, such as the amino acid tryptophan and phytohormone gibberellin in PD regulation (Rinne et al., 2011a; Rutschow et al., 2011). However it is still not clear how these molecules can modify PD, which pathway(s) are involved, and how specific they are compared to the other PD regulators.

1.8 Identification of the \textit{essp8} mutant

The \textit{essp8} mutant was isolated in an unrelated genetic screen developed to identify the gene(s) repressing the expression of seed storage protein (SSP) genes in vegetative tissues (Tang et al., 2008; Lu et al., 2010; Tang et al., 2012a; 2012b; Li et al., 2015). Transgenic Arabidopsis plants, expressing the \textit{β-GLUCURONIDASE} (\textit{GUS}) reporter gene under the control of a seed-specific gene (coding for the \textit{β-CONGLYCININ} β-subunit) promoter from soybean, were generated (\textit{pβCG:GUS}) (Tang et al., 2008). It was previously reported that the promoter can direct seed-specific gene expression in tobacco (Lessard et al., 1993) and Arabidopsis (Hirai et al., 1994). Analysis of the \textit{pβCG:GUS}
transgenic line confirmed specific expression of the GUS reporter gene only in the embryo with no detectable activity in the vegetative tissues (Tang et al., 2008).

Ethyl methanesulfonate (EMS) was used for mutagenizing seeds from the homozygous pβCG:GUS plants. These seeds represented the M₁ generation. M₂ plants were screened for mutations causing the ectopic expression of GUS in the leaves as an indication of the ectopic expression of the seed storage proteins (essp) (Tang et al., 2008). Several mutant lines were isolated in this screen and were characterized (Tang et al., 2008; Lu et al., 2010; Tang et al., 2012a; 2012b). essp8 is one of the mutants isolated and was used in this study. The major goal of this thesis was the molecular characterization of essp8 mutant.

1.9 Thesis objectives

Considering the severe developmental defects and cell identity changes observed in the essp8 mutant seedlings, it was hypothesized that GSL8 plays critical role(s) in cytokinesis and plasmodesmata regulation and is required during early seedling development in Arabidopsis. To test this hypothesis, the following specific objectives were proposed. Please note that some of these goals were developed during the course of this study, as the role of GSL8 became more apparent:

1. To confirm that the GSL8 mutation is indeed the cause of developmental defects and cell identity changes observed in essp8 seedlings.
2. To confirm GSL8 involvement and requirement for cytokinesis and plasmodesmata regulation and to demystify how these functions are linked to the essp8 mutant phenotype.
3. To partially/completely rescue the essp8 mutant phenotype through restoring the callose balance at plasmodesmata using two candidate genes, PDLP5 and AtBG_PPAP.
4. To generate an interactome network for GSL8 by identifying proteins which physically interact with GSL8 in vivo.
CHAPTER 2
2 MATERIAL AND METHODS

2.1 Plant materials and growth conditions

*Arabidopsis thaliana* lines were either in Columbia (Col-0) or Landsberg erecta (Ler) background. Seeds for wild-type Col-0 and Ler were originally obtained from Arabidopsis Biological Resource Center (ABRC). Different T-DNA lines used in this study (Appendix I) were ordered either from ABRC or the European Arabidopsis Stock Center (NASC).

Seeds were sterilized in 70% ethanol for 45 sec, followed by 20% bleach with 0.1% sodium dodecyl sulfate (SDS) for 10 min with gentle shaking. Sterilized seeds were then rinsed with sterile distilled water four times and stored at 4°C in the dark for 48 h for stratification before sowing in soil, or after placing on Murashige and Skoog (MS) (Murashige and Skoog, 1962) agar.

For growing plants in soil after stratification, seeds were sowed into pots containing ProMix-BX (Premier Horticulture, Québec) soil. Plants were grown in either the growth room or chamber for 16 h light at 22°C followed by 8 h dark at 18°C.

For growing plants on MS agar, after surface sterilization, seeds were placed on MS agar containing 1X MS salts; 0.05% 2-N-morpholino-ethanesulfonic acid (MES); 3% sucrose; 0.8% agar; pH 5.8. Appropriate antibiotics or chemicals were added for selection or induction where necessary.

*Nicotiana benthamiana* seeds were obtained from Agriculture and Agri-Food Canada, London, Ontario, and grown in pots containing ProMix-BX under the same growth condition as described for Arabidopsis plants.

2.2 Crossing of Arabidopsis plants

To cross Arabidopsis plants from different genetic backgrounds, flower buds with their petals barely visible were used as pollen recipients. Using fine point tweezers, all the floral organs were carefully removed except for the carpel, which needs to be intact (Altmann et al., 1992). Plants were allowed to recover for 16-24 h. Then the stigma of the
recipient plant was pollinated by placing the pollen from the donor plant on the recipient stigma. The fertilized inflorescence was labeled and seeds from the corresponding silique were collected after being matured. These seeds represent the F₁ generation.

2.3 Histochemical assays

2.3.1 GUS staining

Two-week-old Arabidopsis seedlings were used for detection of ectopic GUS activity. Seedlings were immersed in GUS staining solution (80% distilled water; 20% methanol; 1 mM 5-bromo-4-chloro-3-indolyl glucuronide cyclohexylammonium salt (X-GLUC; Inalco); pH 7-7.5) and vacuumed for 15 min, then incubated at 37°C overnight. Destaining was performed with 75% ethanol followed by incubating at 37°C. Destaining process was repeated, if needed, to achieve a clear background.

2.3.2 Sudan red staining

Sudan red staining for visualizing fatty acid accumulation was performed by incubating three-week-old Arabidopsis seedlings with somatic embryo-like structures in a saturated solution of Sudan red 7B (Sigma) in 70% ethanol at 37°C overnight. Samples were rinsed with 70% ethanol at least three times to obtain a clear background (Bratzel et al., 2010; Tang et al., 2012b).

2.3.3 Aniline blue staining

Aniline blue fluorochrome (Biosupplies Australia PTY Ltd.) is a chemically synthesized fluorochrome which reacts with β-1,3-glucans to give a brilliant fluorescence under UV light. A stock solution of 0.1 mg/ml was prepared in distilled water. Stock solution can be stored at 4°C in the dark for at least one year. Prior to use, the stock solution was diluted 1:3 with 0.1 M K₃PO₄, pH 12.0. Roots of five- or seven-day-old Arabidopsis seedlings were incubated with fluorochrome staining solution (20 µl/root) for 30 min at room temperature then washed with 0.1 M K₃PO₄, pH 12.0 buffer and imaged on a Zeiss Axioscope 2 (Zeiss, Germany) compound fluorescence microscope using a UV laser. The
microscope was integrated with a Nikon DS-Ri2 digital camera using the ACT-1 software (Nikon, Japan).

2.3.4 Propidium iodide staining

Propidium iodide (PI) is a fluorescent intercalating agent with a molecular mass of 668.4 Da that can be used to stain cell walls and nuclei. PI binds to DNA between the bases, with little or no sequence preferences, and with a stoichiometry of one molecule per 4-5 base pairs of DNA. When PI is bound to nucleic acids, the fluorescence excitation and emission maximum are 535 nm and 617 nm, respectively. PI is the most commonly used dye to quantitatively assess DNA content (Suzuki et al., 1997). A typical use of PI in plant biology is to stain the cell wall. Cell membrane is impermeable to PI. Therefore, at low concentration, it is mostly used to identify dead cells. However, by increasing the concentration, it will diffuse into the cell and stains both the cell wall and nuclei.

Depending on the purpose, PI staining can be used for visualizing just cell walls or both cell walls and nuclei. A stock solution of 2 mg/ml of PI (Life Technologies) was prepared in distilled water. The stock solution was stored at 4°C in dark. To stain the cell walls, Arabidopsis roots were dipped in 1 µg/ml PI at room temperature for 3 min and then rinsed with distilled water. To visualize both the cell walls and nuclei, 100 µg/ml PI solution was used. Roots were stained for at least 5 min at room temperature and rinsed twice with distilled water. PI-stained roots were imaged on a Leica TCS SP2 Laser Scanning confocal microscope (Leica, Germany) using 543 nm excitation and 610-630 nm emission.

2.4 Next-generation mapping of essp8

For genetic mapping of the essp8 mutation, M2 plants from Col-0 background were crossed with wild-type plants of the Ler accession. A total of 100 two-week-old seedlings with essp8 dwarf phenotype were selected from the F2 segregating population and used for bulked-segregant analysis (BSA) and rough-mapping. Genomic DNA (gDNA) extracted from these seedlings were pooled and used for BSA with 22 pairs of simple sequence length polymorphism (SSLP) markers (Lukowitz et al., 2000). After
identification of markers linked to the mutation using BSA. gDNA from individual plants were used for PCR-based rough-mapping to narrow down the genomic interval of *essp8* mutation (Appendix II). Pooled gDNA extracted from 64 seedlings were used as template for next-generation sequencing (NGS). NGS library was generated using NGS library preparation kit (Zymo Research). Sequencing was performed on the Illumina MiSeq (Illumina, USA) using 300 nt paired-end sequencing.

### 2.5 Plasmodesmata size exclusion limit assay

For PD SEL assay, wild-type (WT) Col-0 and *essp8* seeds were sown on MS agar plates. Seeds were allowed to germinate and grow in the dark for four or seven days. The hypocotyls of four- or seven-day-old seedlings were used for the assay. Dextran, Alexa Fluor® 488; 3,000 MW, Anionic (ThermoFisher Scientific) and Dextran, Fluorescein, 10,000 MW, Anionic (ThermoFisher Scientific) were dissolved in tris-ethylene diamine tetraacetic acid (Tris-EDTA) buffer, pH 8 at concentrations of 100 mg/ml and 50 mg/ml, respectively. Aqueous solution of dextrans were stored at -20°C and protected from light. Prior to use, the stocks were diluted in Tris-EDTA buffer at a ratio of 1:10.

The hypocotyls were obtained by cutting the seedlings at the hook. For each sample, 1 µl of the diluted probe was injected into the hypocotyl at the cut site using a Hamilton Gastight syringe (Hamilton). Movement of the probe was analyzed right after injection by imaging on a Leica TCS SP2 Laser Scanning confocal microscope (Leica) using 488 nm excitation and 515 to 530 nm emissions.

### 2.6 Generation of stable transgenic plants

Stable transgenic Arabidopsis plants were generated via *Agrobacterium*-mediated transformation using the floral dip method (Zhang et al., 2006). Plants with the genetic background of interest were allowed to bolt. The bolts were then removed to encourage more secondary inflorescences to proliferate from the axillary buds of the rosette. To increase the transformation efficiency, the older siliques were removed right before using the plants for transformation. A single fresh colony of *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986), harboring the binary vector, carrying the gene of
interest, was inoculated into 3 ml liquid Luria-Bertani broth (LB) medium (Bertani, 1951) containing the appropriate antibiotics. Cells were grown for 16-24 h at 28°C shaking at 225 rpm. This feeder culture can be stored at 4°C up to one month and was used to inoculate the transformation cultures. After preparation of liquid LB with appropriate antibiotics, 1 ml of feeder culture was used to inoculate 250 ml LB and grown at 28°C for 16-24 h, shaking at 225 rpm until the bacterial culture reached stationery phase (OD$_{600}$: 1.5-2.0). *Agrobacterium* cells were harvested by centrifugation at 4,000 g for 12 min at room temperature. Next, the cells were resuspended in one volume of freshly prepared 5% (wt/vol) sucrose solution. Just before dipping, Silwet L-77 (Lehle Seeds) was added to the transformation solution at a concentration of 0.02% (vol/vol) and mixed well. The plants were inverted and the aerial parts of the plants were dipped into the *Agrobacterium* cell suspension for 30 sec. The treated plants were gently drained. Dipped plants were wrapped with plastic bags to maintain high humidity. Covered plants were laid down horizontally and kept in the dark for 16-24 h. The cover was removed the next day. The treated plants were transferred back to the growth room. Seeds were collected after 4-5 weeks when the siliques turned brown. These seeds were called T$_1$. Primary transformants were screened on selection media containing cefotaxime (100 µg/ml) and the appropriate amount of selection marker. Cefotaxime was used to inhibit *Agrobacterium* growth on the plate since harvested seeds are heavily covered with *Agrobacteria*. For each selective media plate (150 × 150 × 25 mm), around 2500 seeds were spread. After 7-10 days, transformants could be easily distinguished by their developing healthy green cotyledons, true leaves and roots which extended into the selective medium. Potential transformants were transferred to soil, and confirmed by genotyping (see section 2.7) for their corresponding transgene.

2.6.1 Generation of transgenic constructs

All transgenic constructs were generated using the Gateway™ system (Invitrogen) (Hartley et al., 2000). To create the translational construct for *SHR*, a 3 kb fragment upstream of the 5’ untranslated region (UTR), the 5’-UTR and the genomic sequence except from the STOP codon was amplified from Arabidopsis gDNA (Appendix III), and cloned into the pMDC107 destination vector which does not have a promoter and carries
GFP reporter for C-terminal fusion (Curtis and Grossniklaus, 2003). PDLP5 overexpression construct was generated by amplification of its coding sequence (CDS) from Arabidopsis cDNA (Appendix III), and its cloning into the pEarleyGate103 containing CaMV35S promoter and C-terminally-fused GFP reporter (Earley et al., 2006).

After confirming the constructs by DNA sequencing, the corresponding vectors were transferred to Agrobacterium GV3101 using electroporation (Nagel et al., 1990), and later transformed to plants with floral dipping (Zhang et al., 2006). Transgenic pSHR::SHR:GFP plants were selected on MS agar media containing 50 µg/ml hygromycin B. p35S::PDLP5::GFP overexpression plants were screened on MS agar media containing 12 µg/ml glufosinate. At least ten independent transformants from T_1 generation were propagated to generate T_2 generation. Progeny of individual T_1 plants were screened for the segregation ratio of the transgene. Only T_2 populations which showed a 3:1 segregating ratio, indicating a single insertion of the transgene, were used for further analysis.

2.6.2 Generation of centromere-labeled transgenic plants

Transgenic seeds expressing the centromere labeling construct (pSDS::CENH3::GFP) were obtained from Ghent University (De Storme et al., 2013). Centromeric histone H3 (CENH3) replaces histone H3 in the nucleosomes of active centromeres in all eukaryotic cells (Lermontova et al., 2006). SDS encodes a mitotic cyclin-like protein and is constitutively expressed throughout the whole plant (De Muyt et al., 2009). Therefore, pSDS::CENH3::GFP construct is expressed in all plant cells. The labeling of the centromeres in gsl8 mutants was carried out by crossing GSL8/essp8, GSL8/gsl8-2 and GSL8/gsl8-4 genotypes with homozygous plants expressing pSDS::CENH3::GFP. The F_2 population was used for imaging and quantifying the number of centromeres in different gsl8 mutants.

2.6.3 Generation of miRNA165/6-sensor line

Homozygous seeds for miRNA165/6-sensor line were obtained from Seoul National University (Carlsbecker et al., 2010). The construct used to generate the sensor line
harbors miR165/6 target sequence from PHB under the control of U2 promoter, providing ubiquitous expression, and C-terminally fused to GFP. To test the GFP expression in gsl8 mutants, transgenic plants were crossed with heterozygous GSL8/essp8 plants and F2 seedlings were used for analysis and imaging.

2.6.4 Generation of artificial miRNA transgene constructs

The Web Micro Designer (WMD, http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) was used for designing artificial miRNA (amiRNA) against both GSL8 and GSL10 genes. To generate the XVE::aMIRGSL8/GSL10 construct, first the amiRNA sequence was introduced into pRS300 (Schwab et al., 2006) vector, containing the Arabidopsis miR319a precursor, as the backbone to create aMIRGSL8/GSL10. Then, aMIRGSL8/GSL10 was subcloned into the pDONR221 vector (Invitrogen), and then recombined into pMDC7 Gateway-compatible destination vector (Curtis and Grossniklaus, 2003). In pMDC7, the aMIRGSL8/GSL10 transgene is controlled by an estradiol-inducible promoter. Wild-type Col-0 plants were transformed with the construct using floral dip method (Zhang et al., 2006). Transgenic plants were selected for hygromycin B resistance (see section 2.6.1). T2 transgenic seeds were sown on MS agar media containing 100 µM β–estradiol or Dimethyl sulfoxide (DMSO) as a mock control.

2.7 Polymerase chain reaction-based genotyping

PCR-based genotyping was used to identify homo and heterozygous plants for all T-DNA insertion lines. gDNA was isolated using a modified plant gDNA extraction protocol with cetyltrimethyl ammonium bromide (CTAB) (Tang et al., 2008). PCR was performed using both allele-specific and T-DNA insertion-specific primers (Appendix I and IV). EMS-based mutants were genotyped by PCR followed by sequencing to detect the single nucleotide polymorphism (SNP) of interest. Heterozygous plants were used for further analysis if homozygous mutations were lethal.

Transgenic plants were confirmed by testing the integration of the transgene by PCR with gene-specific and vector-specific primers.
2.8 Gene expression analysis

For quantitative reverse transcription-PCR (qRT-PCR), total RNA was isolated from ~100 mg of plant tissue using the RNeasy Mini Kit (Qiagen). The High Capacity cDNA Reverse Transcription kit (ABI) was used to reverse transcribe the total RNA into cDNA with random primers from the kit. qRT-PCR was performed using the SsoFast EvaGreen Supermix kit (Bio-Rad Laboratories, Inc.) with the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc. USA). The data shown in the figure are the average of three technical and three biological replicates. GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH) was used as the internal reference (Czechowski et al., 2005). PCR primers used in qRT-PCR are listed in Appendix V.

2.9 Förster resonance energy transfer

Förster resonance energy transfer (FRET) is one of the best methods available to identify and quantify protein-protein interactions. In FRET, the energy from an excited state donor fluorophore is transferred to a nearby acceptor, and it occurs only if the emission band of the donor displays overlapping spectra with the absorption band of the acceptor (Kaminski et al., 2014). FRET level can be quantified from potentially-interacting proteins using different methods including acceptor photobleaching, fluorescence lifetime and polarization resolving imaging (Van Munster and Gadella, 2005; Jares-Erijman and Jovin, 2006; Lakowicz, 2006).

To test the interaction of GSL8 with candidate partners, their CDSs were amplified from Arabidopsis cDNA and cloned into pEarleyGate101 and pEarleyGate102 (Earley et al., 2006) using Gateway system (Invitrogen) to generate YFP- and CFP-fusion proteins, respectively (Appendix III). Four-week-old N. bentamiana leaves were co-infiltrated, as described previously (Sparkes et al., 2006), with two constructs expressing GSL8-YFP and the candidate interactor fused to CFP. The reciprocal combinations were also tested. Three days post infiltration (dpi), the FRET between two proteins was quantified using acceptor photobleaching method by imaging on a Leica TCS SP2 Laser Scanning confocal microscope (Leica). Images of the CFP fluorescent, for donor protein, and YFP fluorescent images, for acceptor proteins, were captured using 458 nm excitation and 465
to 505 nm emissions, and 514 nm excitation and 525 to 600 nm emission, respectively. The fluorescence of the CFP and YFP channels were scanned before and after bleaching. Bleaching of the acceptor protein fluorescence was performed using 100% excitation of 514 nm beam for 50 frames. The energy transfer efficiency between the two potentially-interacting proteins was measured based on the fluorescence intensity change in the donor and acceptor, before and after photobleaching using following equation:

\[
\text{FRET efficiency}\% = \frac{(\text{Donor postbleaching} - \text{Donor prebleaching})}{\text{Donor postbleaching}}
\]

\[0 < \text{FRET efficiency} < 100\]

Three independent experiments with at least three biological replicates for each were used to calculate FRET efficiency.

### 2.10 Microscopy and image analysis

Images were captured by a Nikon SMZ1500 (Nikon) dissecting or Zeiss Axioscope 2 (Zeiss) compound light microscopes which were integrated with a Nikon DS-Ri2 digital camera using the ACT-1 software (Nikon). Nikon dissecting scope optical ranges varied between 0.75 and 11.5X, and the compound Zeiss microscope was used with 20 and 40X objectives. TIFF format at a resolution of 3840 x 3072 pixels was used for capturing all images.

### 2.11 Statistical analysis

In all cases, no less than three plants of each genotype per treatment were used to calculate the means. Pair-wise comparison of the means was done using a Student’s t-Test assuming unequal variance. Unless otherwise stated, in all cases “*” and “**” symbols represent the 95% and 99% confidence interval of the means, respectively.
CHAPTER 3
3 RESULTS

3.1 Map-based cloning of the essp8 mutation

As mentioned in section 1.8, the essp8 mutant was identified in an unrelated genetic screen of an EMS mutant population that was intended to identify repressors of SSPs in Arabidopsis seedlings. The M2 segregating population was screened to isolate mutant lines showing distinct ectopic GUS expression pattern in their vegetative tissues (Tang et al., 2008; Lu et al., 2010; Tang et al., 2012a; 2012b; Li et al., 2015).

3.1.1 Morphological phenotype of essp8 seedlings

The essp8 mutant was accidently identified in a screen for mutations affecting repression of seed storage protein genes. essp8 seedlings exhibit an interesting phenotype with several developmental defects including dwarfism, formation of abnormally-developed cotyledons and true leaves, reduced growth of the root and hypocotyl, and generally delayed development compared to wild-type Col-0 (Fig. 3.1A-D). Segregation analysis showed reduced transmission of the mutant allele in essp8 resulted in deviation from the expected 25% homozygous mutant seedlings for a normal recessive single locus Mendelian segregation (Table 3.1). Reduced transmission of the mutant allele can be due to embryo lethality as ~5% of the homozygous seeds were aborted and failed to germinate (Table 3.1). The abortion of essp8 embryos was further confirmed by phenotypic analysis of the siliques from a heterozygous parent where ~25% of the seeds displayed defective phenotype as being smaller, darker and shrunk compared to wild-type seeds (Table 3.2) (Fig. 3.1E-F).

The essp8 mutation is lethal in most of the mutant seedlings (~90%), leading to complete death of the seedlings after three weeks (Fig. 3.2A-C). However, it can induce formation of somatic embryo-like structures in the seedlings which survive longer at a low penetrance (~10%) (Fig. 3.2D-F). The somatic embryo-like structures were confirmed to be true embryos using GUS staining for ectopic GUS expression (Fig. 3.3A-B), and Sudan red staining for accumulation of seed storage-specific triacylglycerols (Fig. 3.3C-D). Screening different segregating population for essp8 mutation, including F2 and back cross 2 (BC2),
Figure 3.1 Morphological phenotype of the *essp8* mutant. (A-D) Comparison of seedling phenotypes of wild-type Col-0 (A) and *essp8* mutants (B-D). (B-C) Fifteen-day-old *essp8* seedlings are very small and exhibit abnormally shaped true leaves (B) (white arrowheads). The surface of the *essp8* cotyledon epidermis is undulated (C) (white arrowheads). (D) The *essp8* roots and hypocotyls are shorter and thicker compared to the WT Col-0 (white arrowheads). (E-F) Morphological analysis of siliques from a heterozygous parent showed that the *essp8* mutation can cause formation of defective seeds (white arrowheads) (E) and seed lethality (F). Scale bars: A-E = 1 mm, F = 500 µm
Table 3.1 Segregation of homozygous *essp8* seedlings in the progeny of selfed *ESSP8/essp8* heterozygous plants

<table>
<thead>
<tr>
<th></th>
<th>Number of plants tested</th>
<th>Percentage of homozygous seedlings</th>
<th>Percentage of non-germinating seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>essp8</em>-1</td>
<td>132</td>
<td>15.9</td>
<td>9.1</td>
</tr>
<tr>
<td><em>essp8</em>-2</td>
<td>112</td>
<td>21.4</td>
<td>8</td>
</tr>
<tr>
<td><em>essp8</em>-3</td>
<td>142</td>
<td>16.2</td>
<td>4.9</td>
</tr>
<tr>
<td><em>essp8</em>-4</td>
<td>132</td>
<td>19.7</td>
<td>5.3</td>
</tr>
<tr>
<td><em>essp8</em>-5</td>
<td>125</td>
<td>20</td>
<td>4.8</td>
</tr>
<tr>
<td><em>essp8</em>-6</td>
<td>123</td>
<td>22.8</td>
<td>4.1</td>
</tr>
</tbody>
</table>
Table 3.2 The percentage of defective seeds in one silique from selfed *ESSP8/essp8* heterozygous plants

<table>
<thead>
<tr>
<th></th>
<th>Number of seeds in one silique</th>
<th>Percentage of defective seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>essp8-1</em></td>
<td>34</td>
<td>23.5</td>
</tr>
<tr>
<td><em>essp8-2</em></td>
<td>34</td>
<td>26.4</td>
</tr>
<tr>
<td><em>essp8-3</em></td>
<td>18</td>
<td>22.2</td>
</tr>
<tr>
<td><em>essp8-4</em></td>
<td>28</td>
<td>21.4</td>
</tr>
<tr>
<td><em>essp8-5</em></td>
<td>23</td>
<td>21.7</td>
</tr>
<tr>
<td><em>essp8-6</em></td>
<td>27</td>
<td>25.9</td>
</tr>
</tbody>
</table>
Figure 3.2 The *essp8* mutation causes somatic embryo formation with an incomplete penetrance.

(A-C) Developmental defects are observed in *essp8* mutant seedlings during the first three weeks after germination. It was also revealed that the *essp8* mutation is lethal in ~90% of the seedlings and leads to seedlings’ death. (D-F) Around 10% of *essp8* seedlings show ectopic cell proliferation and formation of somatic embryo-like structures (red arrowheads) after two (E) and three weeks (F). Scale bars = 1 mm.
Figure 3.3 *essp8* mutant seedlings form somatic embryos.

(A-B) Histochemical GUS assay and (C-D) Sudan red staining confirmed ectopic *GUS* expression (red arrowheads) and accumulation of seed storage-specific triacylglycerols (white arrowheads), respectively, in four-week-old *essp8* seedlings showing somatic embryo-like structures. Scale bars = 1 mm.
showed the same results (data not shown), confirming that *essp8* is a single recessive mutation inducing dwarfism, somatic embryo formation and seed abortion.

### 3.1.2 Next-generation mapping of *essp8* locus

In order to map the *essp8* mutation, M₂ *essp8* heterozygous plants were crossed with WT plants of the Ler accession to generate a segregating F₂ mapping population. Using a small mapping population of 100 F₂ plants, BSA was performed with 22 pairs of SSLP markers (Lukowitz et al., 2000). BSA successfully located the *essp8* mutation on the bottom arm of chromosome II between the nga1126 and nga168 markers (Fig. 3.4A). Further mapping using more SSLP markers (Appendix II) roughly narrowed down the *essp8* mutation to a genomic interval of about 729 kb, covered by the F11F19 and F13M22 BAC clones (Fig. 3.4B).

To identify the SNP, caused by EMS in *essp8*, an NGM approach was used (Austin et al., 2011). After sequencing the pooled genomic DNA isolated from 64 two-week-old *essp8* F₂ seedlings, a SNP desert region, a non-recombinant block caused by linkage to the recessive mutation of interest, was detected on the bottom arm of chromosome II (Fig. 3.5). This result was consistent with both the BSA and rough mapping data. By combining the results from rough-mapping and NGS, all non-synonymous SNPs, caused by EMS, were identified within the candidate region on chromosome II using a web-based tool (http://bar.utoronto.ca/NGM/). Only one EMS-induced point mutation (GC to AT) (Kim et al., 2006) in the AT2G36850 (*GSL8*) was detected. The mutation disrupts a splice site of *GSL8* (Fig. 3.6).

According to gene structure modeling, *GSL8* is a large gene spanning a genomic region of 14,433 bp, consisting of 50 exons, and a coding sequence of 5712 bp (Fig. 3.7A). In *essp8*, the GC to AT substitution disrupts the splice site at intron 22. The retention of intron 22 alters the *GSL8* cDNA and results in introduction of a premature STOP-codon (Fig. 3.7B).

*GSL8* encodes a large integral membrane protein with 1904 amino acids and is predicted to form sixteen transmembrane helices (Tsirigos et al., 2015). The transmembrane domains
Figure 3.4 Rough mapping of the *essp8* mutation.

(A) Using BSA, the *essp8* mutation was located on the bottom arm of chromosome II between the nga1126 and nga168 markers (green square). (B) The position of the *essp8* mutation was narrowed down to a genomic interval of 729 kb, covered by the BAC clones F11F19 and F13M22, on chromosome II. The numbers of recombination events out of the total number of chromosomes examined (200) are indicated.
**Figure 3.5** Identification of the *essp8* mutation using NGM.

NGM revealed one SNP desert region on the bottom arm of chromosome II (red star). The bars represent the natural polymorphism between wild-type Col-0 and Ler accessions, used to generate the F₂ mapping population.
Figure 3.6 Detection of SNPs within the candidate region for the *essp8* mutation.

The combination of rough mapping and NGM results identified one SNP caused by EMS within the candidate region (between the red lines) on chromosome II. The GC to AT mutation is located at an intron splice site of AT2G36850 (*GSL8*).
**Figure 3.7 GLUCAN SYNTHASE-LIKE 8 (GSL8) genomic and protein structure.**

(A) GSL8 consists of 50 exons and has a coding region of 5712 bp (GenBank Accession number GQ373182). The position of T-DNA insertions (yellow triangles) and EMS-induced mutation (red triangle) are indicated. (B) The splice site at intron 22 is disrupted in *essp8*. The retention of intron 22 in *essp8* establishes a premature STOP-codon downstream of exon 22. (C) GSL8 encodes a large transmembrane protein. GSL8 has a large cytoplasmic central loop between the transmembrane domains. The *essp8* mutation results in the truncation of the GSL8 protein at the fifth cytoplasmic helix, indicated by the red line. (D) GSL8 secondary structure is predicted to mostly consist of helices and loops (Källberg et al., 2012).
are clustered into two N-terminal and C-terminal regions leaving a large hydrophilic central loop within cytoplasm (Fig. 3.7C). In essp8, introduction of the premature STOP-codon results in the truncation of the GSL8 protein at the fifth cytoplasmic helix (Fig. 3.7C). Based on RaptorX protein modeling (Källberg et al., 2012), GSL8 protein secondary structure is predicted to consist of 62% helices, 8% beta sheets and 29% loops (Fig. 3.7D).

3.1.3 essp8 is allelic to GSL8

To confirm that ESSP8 is indeed allelic to GSL8, four different T-DNA insertion lines, SALK_11500 (gsl8-1), SALK_109342 (gsl8-2), SAIL_21_B02 (gsl8-3) and SALK_098374 (gsl8-4), where the T-DNA is located either upstream or downstream of the essp8 EMS mutation, were obtained from ABRC (Fig. 3.7A) (Appendix I).

Heterozygous plants were identified by PCR-based genotyping, and the segregation ratio of the homozygous plants with essp8-like phenotype was determined. Homozygous T-DNA mutant seedlings for all four insertion lines exhibited dwarfism as that of essp8. Similarly, the cotyledon and true leaves were thick and abnormally-developed (Fig. 3.8). Analysis of the root morphological phenotype in gsl8 mutants revealed severe defects in their root tissue patterning as they have stunted roots with bloated cells, as well as short, swollen and often branched root hairs (Fig. 3.9). Similar to essp8, the segregation ratio of homozygous T-DNA mutants showed deviation from the expected 25% (data not shown), providing initial evidence that gsl8 mutation can cause embryo lethality.

To further confirm that ESSP8 is an allele of GSL8, an allelism test was performed. Heterozygous plants for three different T-DNA insertion lines (GSL8/gsl8-1, GSL8/gsl8-3 and GSL8/gsl8-4) were crossed with essp8 heterozygous plants (GSL8/essp8). F1 seedlings that were heterozygous for two different mutant alleles of GSL8, i.e., one of the T-DNA insertions and the EMS mutation (gsl8-1/essp8, gsl8-3/essp8 and gsl8-4/essp8), recapitulated the morphological phenotype of homozygous gsl8 and essp8 mutants (Fig. 3.10). The genotype of the seedlings was confirmed using PCR-based genotyping for T-DNA insertion and sequencing for EMS mutation (data not shown). The genotyping results proved that the seedlings with essp8-like phenotype were
Figure 3.8 Morphological phenotypes of gsl8 T-DNA mutant seedlings compared to essp8. (A) Ten-day-old essp8 seedling phenotype, (B-E) phenotypes for gsl8 T-DNA mutants of the same age: SALK_11500 (gsl8-1) (B), SALK_109342 (gsl8-2) (C), SAIL_21_B02 (gsl8-3) (D) and SALK_098374 (gsl8-4) (E). Each of the mutant lines contains a T-DNA insert upstream or downstream of the essp8 mutation. gsl8 T-DNA insertion mutants exhibited similar phenotypes by forming dwarf seedlings, developing thick cotyledon and true leaves as well as short and thick hypocotyl and roots. (F) Ten-day-old wild-type Col-0 seedling is shown as control. Scale bars = 500 µm
Figure 3.9 Analysis of the root morphological phenotype of *gsl8* mutants showing severe defects in root tissue patterning.

(A) Ten-day-old *gls8* mutant seedlings (*essp8*, *gsl8-1* and *gsl8-2*) develop stunted roots compared to the wild type. (B-I) Comparison of the root phenotype between five-day-old wild-type and *gsl8* mutant seedlings at the root tip and elongation zone of the root. (C-E) *gsl8* mutants showed abnormally-developed root tips with having bloated cells. (G-I) Morphological phenotype of the elongation zone of the primary roots in *gsl8* mutants revealed formation of short, swollen, and often branched root hairs. Scale bars: A = 200 μm, B-I = 50 μm
Figure 3.10 Allelism test confirms that ESSP8 is an allele of GSL8. (A) GSL8/essp8 seedlings show no recognizable phenotypic defects. (B-D) Ten-day-old seedlings that were heterozygous for two different mutant alleles of GSL8, essp8 mutation and gsl8-1 (B), gsl8-3 (C) and gsl8-4 (D) T-DNA insertions, respectively, exhibited a phenotype similar to that of homozygous gsl8 mutants. Scale bars = 500 µm
heterozygous for both mutant alleles. Taken together, the morphological phenotype of four different T-DNA insertion lines for \textit{GSL8} and the allelism test demonstrated that \textit{ESSP8} is indeed a new allele of \textit{GSL8} (AT2G35850) and, therefore, the cause of the developmental defects in Arabidopsis seedlings.

### 3.2 Phylogenetic analysis of Arabidopsis GSLs

To comprehend the relationship among the twelve members of the GSL family in Arabidopsis, a phylogenetic tree of the family was generated. Putative protein sequences of Arabidopsis GSL1-12 and callose synthase from \textit{Phytophthora infestans}, as outgroup, were obtained from the national center for biotechnology information (NCBI) at the time (Appendix VI). A phylogenetic tree was generated with MEGA6 (Tamura et al., 2013) program, using the maximum likelihood method. Alignment of all twelve Arabidopsis GSLs positioned these proteins into three distinct groups (Fig. 3.11). According to the phylogenetic analysis, GSL8 is more closely related to GSL10 as they are clustered into the same subfamily and share 63.7% and 63.4% identities at the DNA and protein sequences levels, respectively. Although there are several lines of evidence suggesting a partial redundancy between the members which belong to the same subfamily (Jacobs et al., 2003; Nishimura et al., 2003; Enns et al., 2005), GSL8 and GSL10 have not yet been shown to be functionally redundant. As was previously predicted (see section 1.2.2), GSL1 and GSL5 were positioned into a different group, reconfirming that GSL1 and GSL5 are different from the other member of the Arabidopsis GSL family.

#### 3.2.1 The cytoplasmic domains of Arabidopsis GSLs are highly conserved

To predict which domain(s) in the GSL proteins are responsible for their enzymatic activity, the amino acid sequences from all twelve members in Arabidopsis were further analyzed by performing an alignment using PRALINE, a web-based tool for multiple sequence alignment (Simossis and Heringa, 2005). It has been classically assumed that the cytoplasmic domain of GSLs contains the catalytic site (Cui et al., 2001; Doblin et al., 2001; Hong et al., 2001a; Østergaard et al., 2002). Multiple sequence alignment of GSLs shows that GSLs’ amino acid sequences are mostly semi- to fully-conserved among all
Figure 3.11 Phylogenetic tree of Arabidopsis GSLs.

The unrooted tree is based on an amino acid alignment of full-length sequences from Arabidopsis (Appendix VII). The evolutionary history was inferred by using the maximum likelihood method. The tree with the highest log likelihood (-62692.0592) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branch points. The tree is drawn to scale, with branch lengths reflecting the number of substitutions per site. There were a total of 1768 positions in the final dataset. The phylogenetic tree was generated with MEGA6 using a bootstrap value of 500.
twelve members of the family with very short non-conserved regions (Appendix VII). As has been predicted, the conserved residues are mainly found in the cytoplasmic domains. The largest number of conserved residues was found in the ninth cytoplasmic loop of GSL8, suggesting that this domain can potentially act as a catalytic and/or interacting site of these enzymes with other proteins or co-factors (Fig. 3.12).

3.3  Callose deposition at both cell plate and PD is decreased in essp8 root

As GSL8 has been suggested to be involved in callose biosynthesis, the effect of the gsl8 mutation on callose deposition at the cell plate and PD was further investigated. Callose accumulation was visualized using a callose-specific dye, aniline blue fluorochrome, in WT Col-0 and essp8 roots. Two T-DNA insertion lines (gsl8-1 and gsl8-2), which contain insertion sites upstream and downstream of the EMS mutation, respectively (Fig. 3.7A), were also analyzed (Fig. 3.13). In the WT Col-0 root tip, bright, linear signals represent the callose deposited at the cell plate in dividing cells (Fig. 3.13A). Furthermore, punctate fluorescent signals were detected on the cell walls in the elongation zone of the roots, root hairs and vascular tissues, which indicate characteristic callose deposition at the PD (Fig. 3.13E). In contrast to the WT Col-0, essp8, as well as gsl8-1 and gsl8-2 roots showed almost no signal from callose either at the cell plate in the root tip (Fig. 3.13B-D) or PD in the elongation zone of the root (Fig. 3.13F-H). Absence of callose deposition was also noticed in the root hairs and vasculature tissues in gsl8 mutants. Based on these findings, it can be concluded that GSL8 is required for proper deposition and accumulation of callose at the cell walls, cell plates and plasmodesmata.

3.4  essp8 is a cytokinesis-defective mutant

Formation of defective cell plate during cytokinesis can cause abortion of cell division. Depending on the stage that mitosis is aborted, separation of the newly-formed nuclei or duplicated chromosomes is disrupted, resulting in generation of multi-nucleated cells or cells with doubled number of chromosomes (Joubès and Chevalier, 2000) (Fig. 1.2).

3.4.1  GSL8 is required for the completion of plant cytokinesis

Callose has already been characterized as an essential component of the cell plate (see
Figure 3.12 The predicted conserved domains of the GSL8 protein.
According to the alignment result (Appendix VII), the conserved residues are mainly located in the cytoplasmic domains. The longest conserved stretch of residues corresponds to the ninth cytoplasmic loop in GSL8, shown in red. The numbers indicate the predicted number of amino acids forming different domains.
**Figure 3.13** Callose deposition in the primary roots of *gsl8* mutants.

Callose accumulation was visualized using aniline blue staining in five-day-old seedlings of WT Col-0, *essp8*, and two T-DNA insertion lines, *gsl8-1* and *gsl8-2*. (A-D) Bright lines (white arrowhead) at the root tip represent callose deposition at the cell plate in WT Col-0 (A) and *gsl8* mutants. (E-H) In the elongation zone of the primary root, callose deposition at plasmodesmata is detected as dots (white arrowheads) in the root hairs, cell wall and vascular tissue in the WT Col-0 (E) and *gsl8* mutants. Scale bars = 100 µm.
To investigate if severe phenotypic defects in *essp8* seedlings are caused by incomplete cytokinesis, different known cytokinesis-defective mutants including *hinkel* (Strompen et al., 2002), *knolle* (Touihri et al., 2011), *keule* (Waizenegger et al., 2000), *korrigan* (Sato et al., 2001) and *stomatal cytokinesis-defective 1 (scd1)* (Falbel et al., 2003) were screened for their morphological phenotypes.

Previous studies have shown that mutations in genes that are required for cytokinesis execution, such as *HINKEL*, *KNOLLE* and *KEULE*, induce formation of multi-nucleated cells which are characterized by having gapped or incomplete cell walls (Assaad, 2001). *HINKEL* encodes a kinesin-like protein which is required for cytoskeletal formation (Strompen et al., 2002). *KNOLLE* and *KEULE* encode a syntaxin and a Sec1/Munc18 proteins, respectively, and are required when vesicles are fusing to form the cell plate (Lukowitz et al., 1996; Waizenegger et al., 2000). *KORRIGAN* belongs to a different class of genes required for cytokinesis. It encodes a membrane-bound endo-1,4-β-glucanase (Sato et al., 2001). Cell wall formation is disrupted in *korrigan* mutants whose mutant seedlings are identified as having cell-wall stubs and swollen cells (Söllner et al., 2002). *SCD1* is involved in vesicle trafficking during cytokinesis (Falbel et al., 2003). Epidermal and guard cells in *scd1* mutants are binucleated and have cell wall stubs, indicating that *scd1* plants are cytokinetically defective (Falbel et al., 2003).

T-DNA insertion lines for all five selected cytokinesis genes were obtained from ABRC (Appendix I). After germinating seeds on MS agar media, the phenotypes of the homozygous seedlings were compared to that of *essp8*. Two of the mutants, *hinkel* and *scd1*, showed more similar phenotype to *essp8* by having small seedlings, fairly short roots and thicker leaves (Fig. 3.1A-C). However, even though *knolle*, *keule* and *korrigan* also form tiny seedlings, they develop fused cotyledons and fail to form true leaves, which are different characteristics compared to *essp8* seedlings (Fig. 3.1D-F). The possibility of somatic embryo formation by cytokinesis-defective mutants was also investigated. Despite the fact that *hinkel* and *scd1* show elevated cell proliferation, and form somatic embryo-like structures, none of them were confirmed to be true somatic embryos by Sudan red staining (data not shown).
To explore if *essp8* mutant seedlings show any secondary effects of cytokinesis defects such as formation of binucleated cells, PI staining was used to visualize the cell wall and nuclei of cells in the elongation zone of roots. Analysis of these cells was performed by confocal microscopy to detect bi- or multi-nucleated cells in which two daughter nuclei do not seem to be separated by a cross-wall. As expected, confocal imaging confirmed the presence of binucleated cells in cytokinesis-defective mutants including *scd1*, *keule*, *korrigan* and *knolle* (Fig. 3.15E-H). Similar to cytokinesis-defective mutants, *gsl8* mutant seedlings including *essp8*, *gsl8-1* and *gsl8-2* have cells with more than one nucleus, suggesting that *gsl8* can also be defined as a cytokinesis-defective mutant (Fig. 3.15B-D). A very frequent defect observed in *essp8* roots was enlarged disorganized cells with abnormal shapes, compared to the WT Col-0, which could not be detected in the cytokinesis mutants, indicating that *essp8* morphological and developmental defects are only partially caused by cytokinesis impairments.

### 3.4.2 Cytokinesis impairment in *essp8* induces ectopic endomitosis

Identification of binucleated cells in somatic tissue of *essp8* root organs prompted me to assess the possibility of another cytokinesis-related defect. To investigate whether *essp8* mutation also induces ectopic endomitosis in the root, the expression of a centromere-labeling construct, *pSDS::CENH3::GFP* (De Storme et al., 2013), in the *gsl8* background was analyzed. Since this construct is expressed in all plant tissues and organs, the absolute number of chromosomes can be quantified *in vivo* by detection of fluorescent GFP in the tissue of interest.

The primary roots of homozygous *essp8*, *gsl8-2* and *gsl8-4* mutant seedlings expressing *pSDS::CENH3::GFP* were used for analysis. The diploid status of the root cells in WT Col-0 was confirmed by detection of 5 to 10 centromeric dots (Fig. 3.16A). Interestingly, the *essp8*, *gsl8-2* and *gsl8-4* root cells have nuclei carrying higher number of chromosomes (ranging from 11 to 15), indicating the presence of triploid, and potentially polyploid cells in these mutants (Fig. 3.16B-D). A comparison of the distribution pattern as well as number of centromeres between WT Col-0 and *gsl8* mutants revealed a significant difference for all three *gsl8* mutants (*essp8*, *gsl8-2* and *gsl8-4*) compared to WT, whereas the difference among the *gsl8* mutants was found not to be significant (Fig. 3.16E).
Figure 3.14 Morphological phenotypes of cytokinesis-defective mutants.

(A-F) Comparison between *essp8* (A) and cytokinesis-defective mutant seedlings: *hinkel* (B), *scd1* (C), *knolle* (D), *keule* (E) and *korrigan* (F). All cytokinesis-defective mutants develop dwarf seedlings, but *hinkel* and *scd1* are morphologically more similar to *essp8* with having abnormally-developed cotyledons and true leaves (B), and thicker roots and hypocotyls (C) (red arrowheads). Scale bars = 1 mm
Figure 3.15 Formation of binucleated cells in the primary roots of gsl8 and cytokinesis-defective mutants.

(A-D) Comparison of the number of nuclei per cell in the elongation zone of the root between wild-type Col-0 (A) and gsl8 mutants: essp8 (B), gsl8-1 (C), gsl8-2 (D). (E-H) The primary roots of cytokinesis-defective mutants harbor binucleated cells: scd1 (E), keule (F), korrigan (G) and knolle (H). Examples of binucleated cells are indicated by white arrowheads in gsl8 and cytokinesis-defective mutants. Propidium iodide was used to stain both nuclei and cell walls. Scale bars = 20 µm
Therefore, it was concluded that the *essp8* mutation can induce not only ectopic endomitosis in reproductive cells (previously shown by De Storme et al., 2013), but also in the somatic cells in the root at early stages of development.

Ectopic polyploidization and endomitosis in *essp8* seedlings were further confirmed by detection of enlarged cells. In contrast to wild-type roots which always show homogeneously sized cells containing equally sized single diploid nuclei (Fig. 3.15A), *gsl8* roots have enlarged abnormally-shaped cells with polyploid nuclei (Fig. 3.15B-D). Interestingly, the frequency of these enlarged polyploid cells increases with age. Analyzing the root cells and nuclei at five and ten days using the centromere labeling construct revealed that, at five days, *essp8* root tissues often contained scattered single enlarged endomitotic and/or polyploid cells, as only five days later, clusters of enlarged polyploid cells could be found (Fig. 3.17A-D). This result indicates that with age, as the seedlings start deteriorating, the polyploidization defects significantly increase (Fig. 3.17E).

### 3.5 GSL8 regulates symplastic connectivity through plasmodesmata

Plant cells are interconnected with each other via narrow channels of PD, facilitating cell-to-cell communication (Rinne and van der Schoot, 1998). Callose deposition at the PD plays an important role in symplastic movement of various substances (see section 1.1.6) (Levy et al., 2006; Lucas et al., 2009; Xu and Jackson, 2010; Maule et al., 2011; Zavaliev et al., 2011; Xie et al., 2012). Callose deposition is a highly-regulated dynamic process, which is required for adjustment of PD SEL in response to endogenous and exogenous signals (De Storme and Geelen, 2014).

#### 3.5.1 Loss of GSL8 induces an increase in plasmodesmata size exclusion limit

Since aniline blue staining results have already indicated the requirement of GSL8 for callose deposition at both cell-plate and PD (Fig. 3.13), it was postulated that lack of callose deposition at the PD in *essp8* causes an increase in SEL of the PD. To test this hypothesis, alteration of cell-to-cell symplastic movement was investigated in the *essp8* hypocotyls. Two different fluorescent probes, Alexa Flour and fluorescein (see section 2.5), with different sizes (3 kDa and 10 kDa, respectively) were used as markers
Figure 3.16 Comparison of centromere numbers in the primary roots of wild-type Col-0 and gsl8 mutants shows an increase in gsl8 mutants. (A-D) The number of centromeres per nucleus in the primary roots in the WT Col-0 (A) and gsl8 mutants: essp8 (B), gsl8-2 (C) and gsl8-4 (D). Quantification revealed up to ten centromeres per nucleus in the WT Col-0, reflecting a basic diploid status, whereas all three gsl8 mutants have more centromeric dots indicating the occurrence of endomitotic polyploidization. Sample nuclei are marked by circles. (E) Quantification of centromere numbers in the WT Col-0 and gsl8 mutants shows a significant difference. The boxes signify the upper (purple) and lower (green) quartiles, and the median is represented by a short black line within the box for each plant line. The upper and lower “whiskers” represent the entire spread of the data. Data were acquired from at least ten cells in an individual seedling and three seedlings per genetic background were examined. Dotted lines show which samples were compared pairwise and significant differences at P < 0.01 using Student’s t-Test are indicated by **. Scale bars = 10 μm
**Number of centromeres per nucleus**

Col-0  gs8-2  gs8-4  essp8

(E)
Figure 3.17 Comparison of polyploid cells in *essp8* mutant at different ages shows an increase in ploidy level with age.

(A-B) The number of centromeres in the primary root cells of five- and ten-day-old wild-type Col-0, and (C-D) *essp8*, respectively. (E) In *essp8*, the number of endomitotic cells significantly increases with age and clusters of polyploid cells with higher number of chromosomes are formed in ten-day old (D) compared to five-day-old primary roots (C). (E) Quantification of centromere numbers shows no significant difference in WT Col-0 seedlings. The data obtained from at least ten cells in each individual seedling with three biological replicates. Dotted lines show pairwise significant differences at **P < 0.01 using Student’s t-Test. Scale bars = 10 μm.
to monitor passive cell-to-cell diffusion.

Fluorescent probes were separately injected to the hypocotyls after the seeds have been germinated for four days in the dark (Fig. 3.18A-B). Diffusion of fluorescent signal, from the point of initial injection, was assayed by measuring their distance between the injection site and the furthest detected signal. For the smaller probe, in both WT and essp8, the fluorescent signal was detected at the site of injection and surrounding cells (Fig. 3.18C-D); however, the distance of its movement was significantly longer in essp8 (Fig. 3.18G). In contrast, the larger probe (fluorescein) was only detected at the site of injection in nearly all cases in WT. Only a few surrounding cells showed a dim fluorescent signal, indicating its minor diffusion in the WT hypocotyls (Fig. 3.18E). Contrary to the WT, essp8 hypocotyl cells showed strong fluorescent signal in many more surrounding cells (Fig. 3.18F) as well as significantly longer distance of movement (Fig. 3.18G).

Diffusion patterns of Alexa Fluor in both WT and essp8 hypocotyls indicate that its size (3 kDa) is below the SEL. Lack of fluorescein diffusion in the WT suggests that 10 kDa is possibly beyond the SEL of hypocotyl. On the contrary, the fluorescent signal in surrounding cells in essp8 was detected significantly further away from the injection sites compared to the WT, which indicates that larger molecules were able to spread into neighboring cells in the essp8 hypocotyl. These results suggest that in the essp8 mutants, reduction of callose deposition at the PD is likely to be associated with an increase in SEL and, therefore, alteration of symplastic connectivity between hypocotyl cells.

3.5.2 Dysregulation of SHORT ROOT movement through PD in the essp8 roots

Arabidopsis root is composed of concentric cell layers including epidermis, cortex and endodermis, which surround the stele that comprises the vascular tissues and pericycle (Dolan et al., 1993). To gain a better understanding of the downstream effects of PD defects in the essp8 mutants on PD-mediated intercellular signaling during root development, SHR, an important developmental regulators in roots, was used as a molecular marker (see section 1.5.2). During root development in Arabidopsis, the endodermis, middle cortex (MC), and cortex are formed by timely and spatially regulated
Figure 3.18 Quantitative measurement of the fluorescent probes movement in *essp8* hypocotyl.

(A-B) The hypocotyls of wild-type (A) and *essp8* (B) etiolated seedlings were used for the assay. (C-F) Using two fluorescent probes, Alexa Fluor (3 kDa) (C-D) and fluorescein (10 kDa) (E-F), the symplastic connectivity was quantitatively measured in wild-type (C and E) and *essp8* (D and F) hypocotyls. The injection sites are shown by single asterisks. (G) For both the Alexa Fluor and fluorescein probes, the distance of the probes’ movement in WT Col-0 was significantly less than that in *essp8*. In all cases, values reported are the mean ± S.E (n = 5). The double asterisks denote significant differences (Student’s t-Test, **P < 0.01). Scale bars: A-B = 1 mm, C-F = 100 µm.
periclinal cell divisions. The formation of endodermis and cortex occurs continuously by parallel division of the cells surrounding the QC at the root tip, and is mediated by the activity of two transcription factors SHR and SCR (Di Laurenzio et al., 1996; Helariutta et al., 2000; Wysocka-Diller et al., 2000). It has been previously suggested that the intercellular movement of SHR is PD-mediated (Vatén et al., 2011).

essp8 mutant seedlings display severe defects in root tissue patterning with cell identity change and increased number of cell layers resembling the phenotype of SHR ectopic expression. Since it was already shown that the essp8 mutation causes reduced callose accumulation at the PD (Fig. 3.13E-H) accompanied by an increase in PD SEL (Fig. 3.18G), it was hypothesized that the movement of SHR is altered during root development as a consequence of the PD defects in essp8.

To determine whether the root phenotype in essp8 is, at least partially, SHR-dependent, the pSHR::SHR:GFP expression construct was introduced into WT Col-0 and essp8 heterozygous plants. In five-day-old WT seedlings, SHR is localized into nuclei and cytoplasm in the stele cells, while in the neighboring cell layers including the QC, CEI, and endodermis, SHR specifically resides in the nucleus (Fig. 3.19A). Contrary to the WT, in essp8 five-day-old seedlings, SHR-GFP fusion protein signal was detected in both nucleus and cytoplasm in the stele cells and the neighboring layers including the QC, CEI and endodermis, as well as cortex (Fig. 3.19B). Although severe defects in root tissue patterning in ten-day-old essp8 roots make the recognition of different cell layers and tissues challenging, the SHR-GFP fusion protein signal was detected in the outer cell layers, indicating an increase in intercellular movement of SHR in the essp8 roots compared to the wild type (Fig. 3.19C-D).

This result reconfirms that SHR cell-to-cell trafficking is PD-mediated. Defects in PD aperture in the gsl8 mutants interfere with the highly-regulated movement of SHR. Dysregulation of SHR symplastic movement leads to its ectopic localization to the cortex and outer cell layers, as well as ectopic subcellular localization into the cytoplasm in the embryonic roots of gsl8 mutant. Similar results were obtained using two other gsl8 mutants (gsl8-1 and gsl8-2) (data not shown), which further confirmed that gsl8 mutants are PD-defective.
Figure 3.19 Symplastic movement of SHR is altered in *essp8*.

(A-B) Localization of SHR-GFP fusion proteins in five-day-old wild-type Col-0 (A) and *essp8* (B) primary roots. SHR movement and subcellular localization are dysregulated in *essp8* roots as early as five days. (C-D) Localization of SHR-GFP fusion protein in the root tips of ten-day-old wild-type Col-0 (C) and *essp8* (D). Dysregulation of SHR movement and its localization become more severe over time as the root tissue pattern deteriorates with age. Roots were stained with PI to visualize the cell wall. Scale bars = 20 µm
3.5.3 The movement of miR165/6 is altered in the essp8 root

It has been shown that the miR165/6 species (see section 1.5.4) are transcribed in the endodermis outside of the stele, where SHR is required for their transcription, and then move from the endodermis into the stele where they target the transcripts of class III homeodomain leucine zipper (HD-ZIP III) family genes (Carlsbecker et al., 2010; Miyashima et al., 2011). To further understand if the defective root tissue patterning in essp8 is, at least partially, the result of an increase in PD SEL and impaired symplastic signaling, miR165/6 activity in the endodermis and stele in essp8 roots was investigated using a ‘miRNA-sensor’ system (Carlsbecker et al., 2010). In this system, lower GFP signal is an indicator of higher miR165/6 activity (see section 2.6.3).

In WT Col-0 roots, the detected GFP signal was weak but consistent in the stele and endodermis in both five- and ten-day-old seedlings, indicating miR165/6 activity in these cell layers as expected (Fig. 3.20A and C). The GFP signal was more obvious in the QC as well as lateral and collumella root cap, confirming the absence of miR165/6 activity. In five-day-old essp8 roots, the miR165/6 activity pattern was similar to that of the WT with weak GFP signal in the stele and endodermis (Fig. 3.20B). However, the detected signal in the QC, lateral and collumella root cap was weaker compared to WT Col-0, suggesting that miR165/6 might also be present and active in these cell layers (Fig. 3.20B). In the ten-day-old essp8 seedlings, miR165/6 was found to be ectopically active in the outer cell layers, which was confirmed by the detection of very weak GFP signals in the stele, endodermis, epidermis, as well as lateral and collumella root cap cells (Fig. 3.20D). Taken together, these results suggest that lack of callose accumulation at the PD in essp8 possibly dysregulates PD-mediated intercellular signaling and, therefore, results in ectopic activity of miR165/6 in the outer cell layers of the root in essp8 mutant.

3.5.4 WUSCHEL symplastic movement might be dysregulated in essp8 shoot apical meristem

WUS (see section 1.5.3) is required for stem cell specification (Laux et al., 1996). WUS activates CLV3 transcription in the adjacent cell layers of SAM by directly binding its
Figure 3.20 Cell-to-cell movement of miR165/6 is dysregulated in *essp8*. miR165/6 activity in five-day-old wild-type Col-0 (A-B) and ten-day-old seedlings (C-D) in wild-type Col-0 (A, C) and *essp8* (B, D) primary roots, respectively. In the WT, the activity of miR165/6 in the stele and endodermis cells shows a weak GFP signal, whereas in the root cap, absence of miR165/6 activity results in a strong GFP signal (A). In five-day-old *essp8* roots (B), the GFP signal in the root cap is weaker compared to the WT. The detected GFP signal in the ten-day-old *essp8* root cap is noticeably weaker compared to the WT (C and D). Scale bars = 20 µm
genomic region (Yadav et al., 2011). Restricted movement of WUS for two cell layers in SAM is critical for stem cell activity and specification. Mislocalization of WUS in the neighboring cells of SAM induces expansion of the stem cells and proliferation of meristematic cells (Yadav et al., 2010). There is supporting evidence that WUS trafficking is more likely to occur through PD (Daum et al., 2014). Given the possibility that movement of WUS in SAM is mediated through PD and the observation of defective PD in essp8 mutants, it was postulated that WUS is mislocalized into adjacent cell layers of SAM in essp8 mutants, leading to up-regulation of CLV3 in the essp8 seedlings compared to the WT.

To address this hypothesis, the expression level of CLV3 was determined by qRT-PCR in wild-type and essp8 seedlings at two different ages (five- and 10-day-old). Interestingly, a significant increase was found in expression of CLV3 in essp8 in both five- and ten-day-old seedlings, with being more significant in ten-day-old seedlings (Fig. 3.21). Taking into consideration that WUS activates CLV3 transcription, the up-regulation of CLV3 in essp8 seedlings indirectly indicates the possibility of WUS ectopic movement and, therefore, ectopic activation of CLV3 transcription. This result suggests that in SAM, similar to RAM, PD defects may cause an alteration in intercellular communication.

3.6 Attempts to rescue essp8 mutant phenotype

To examine whether the reduced callose at the PD and consequently increased SEL of the PD are related to the essp8 defective mutant phenotype, genetic approaches were used to, at least partially, rescue the essp8 phenotype. Based on our current understanding of the PD regulation and identified proteins in PD proteome (Table 1.1), two candidates, AtBG_PPAP (Levy et al., 2007; Zavaliev et al., 2013) and PDLP5 (Lee et al., 2008; Thomas et al., 2008; Lee et al., 2011; Wang et al., 2013) were chosen for the tests to rescue the essp8 mutant phenotype and, specifically, its PD defects.

3.6.1 Knocking out AtBG_PPAP cannot restore callose balance at the PD in essp8

Deposition of callose is a reversible process which occurs both during developmental processes (Rinne and van der Schoot, 1998; Ruan et al., 2004) and in response to stress
Figure 3.21 Analysis of *CLAVATA3* transcript levels by qRT-PCR.

The expression level was normalized to that of *GAPDH*. Error bars indicate standard deviation for three biological replicates. The asterisks denote significant differences using Student’s t-Test, * P < 0.05, ** P < 0.01.
AtBG_PPAP encodes a β-1,3-glucanase (see section 1.4.2) which is associated with PD and is localized to the cell membrane (Levy et al., 2007). It has been previously shown that knocking out β-1,3-glucanase results in an increase in callose accumulation around PD and reduces SEL. Based on these results, it has been proposed that callose turnover, as a result of its synthesis vs. hydrolysis, is responsible for regulating PD SEL (Levy et al., 2007). Therefore, a balance between the activities of β-1,3-glucanases and callose synthases (GSLs) is required to deposit callose at the neck of PD, and regulate PD’s symplastic flow by adjusting the channel size (Fig. 1.5) (Maule et al., 2012).

essp8 is a GSL8 loss-of-function mutant. Therefore, it was assumed that the balance between callose synthases and β-1,3-glucanases is disrupted in essp8, presumably with higher callose hydrolysis activity than callose synthesis. It was hypothesized that knocking out AtBG_PPAP partially restores the balance between the activity of the enzymes hydrolyzing and synthesizing callose, and, consequently, rescues essp8 mutant phenotype and PD defects moderately. To address this hypothesis, a T-DNA insertion line for AtBG_PPAP, atbg-1, was ordered from ABRC (Appendix I). After genotyping, individual plants that were homozygous for the T-DNA insertion were crossed with heterozygous GSL8/essp8 and GSL8/gsl8-1 plants, and F2 population was screened to identify the double mutants (atbg-1 essp8 and atbg-1 gsl8-1). Double mutants were identified using PCR-based genotyping and sequencing to confirm the atbg-1, gsl8-1 and essp8 genotypes. atbg-1 essp8 and atbg-1 gsl8-1 double mutants were identified at the ratios of around 5% and 6% in the F2 population, respectively.

Interestingly, knocking out AtBG_PPAP in essp8 and gsl8-1 genetic backgrounds could not rescue the morphological defects of gsl8 mutants (Fig. 3.22A-D). To investigate whether the atbg-1 mutation can rescue PD defects, the callose accumulation was visualized using aniline blue staining. Callose visualization showed an increase in the amount of callose deposited at PD in atbg-1 compared to the WT, and in atbg-1 essp8 double mutant compared to essp8 single mutant (Fig. 3.22E-H). No obvious change was detected at the cell plate (data not shown). This result indicates that only a minor
elevation of the callose deposited at PD is not sufficient to rescue the PD defects and subsequently, the gsl8 mutant phenotype.

3.6.2 PDLP5 overexpression induces callose deposition at PD

It was been previously suggested that PDLPs (see section 1.4.1) can induce callose deposition at PD and, therefore, might negatively regulate SEL of PD (De Storme and Geelen, 2014). PDLP5 overexpression can elevate plants’ resistance against viral infection, which is achieved through an increase in callose deposition at PD and thus, restricts the symplastic movement of the viral genome MPs (Lee et al., 2008; 2011). It was previously shown that in essp8, callose deposition at PD is reduced (Fig. 3.13F-H), and cell-to-cell communication is impaired (Fig. 3.18), supposedly due to an increase in SEL of PD. Thus, it was hypothesized that PDLP5 overexpression in gsl8 mutant background partially rescues PD defects and, therefore, the altered symplastic signaling.

To test my hypothesis, the p35S::PDLP5:GFP expression construct was introduced into WT Col-0 and GSL8/essp8 heterozygous plants to generate PDLP5-overexpression lines (PDLP5-OE). Wild-type seedlings overexpressing PDLP5 exhibited yellowish color compared to the wild-type Col-0 (Fig. 3.23A-B), consistent with a previously published observation (Lee et al., 2011). PDLP5 overexpression did not rescue the gsl8 phenotypic defects in essp8 background (Fig. 3.23C). To confirm that PDLP5 in transgenic plants is indeed localized at the PD, its localization was examined in the roots of five-day-old seedlings. PDLP5-GFP signals were detected at punctate particles at the cell membrane of root cells, suggested to be associated with the PD apertures, which is consistent with previous reports on PDLP5 (Lee et al., 2011) and its homolog, PDLP1 (Thomas et al., 2008) (Fig. 3.23D-E). Additionally, in essp8 PDLP5-OE root cells, PDLP5-GFP signal could occasionally be detected inside the cell periphery (Fig. 3.23F), suggesting that PDLP5 localization might be disrupted in essp8 plants.

To determine if PDLP5 can induce callose deposition at the PD, aniline blue staining was used to visualize the callose accumulated at the PD in the PDLP5-OE lines in wild type and essp8 backgrounds. Staining of the primary roots in seven-day-old seedlings showed
Figure 3.22 Phenotypes of *atbg-1* *gsl8* double mutants.

(A-D) The morphological phenotypes of *atbg-1* (A), *essp8* (B), *atbg-1* *essp8* (C) and *atbg-1* *gsl8*-1 (D) two-week-old seedlings. *atbg-1* seedlings do not exhibit distinguishable phenotypic defects. Knocking out *AtBG_PPAP* in the *gsl8* background does not rescue the defective phenotype of *gsl8* mutants compared to an *essp8* single mutant. (E-H) Visualization of callose deposition at the PD using aniline blue staining in seven-day-old primary roots of wild-type Col-0 (E), *atbg-1* (F), *essp8* (G) and *atbg-1* *essp8* (H) seedlings. Callose deposited at the PD is indicated by white arrowheads. Scale bars: A-D = 1mm, E-H = 50 µm
that PDLP5 overexpression in the WT background induces an increase in callose accumulation at the PD (Fig. 3.23G-H), while no change was detected in essp8 PDLP5-OE roots (Fig. 3.23I).

3.6.3 PDLP5 and AtBG_PPAP regulate PD’s SEL

To further elucidate if modulation of callose deposition at the PD, induced by PDLP5 overexpression and/or knocking out AtBG_PPAP, can indeed affect the SEL, cell-to-cell communication was tested using the SEL assay (see section 2.7). Measuring the extent of fluorescein probe movement in seven-day-old hypocotyls confirmed that an increase in callose deposition, induced by AtBG_PPAP knockout or PDLP5 overexpression, modulates the SEL in atbg-1 and PDLP5-OE compared to wild-type Col-0. Cell-to-cell movement of probe showed a reduction in both atbg-1 and PDLP5-OE hypocotyls compared to wild-type Col-0, with PDLP5-OE displaying a significant decrease. Knocking out AtBG_PPAP in essp8 background could significantly decrease the SEL. However, no difference could be detected between essp8 PDLP5-OE and essp8 seven-day-old hypocotyls (Fig. 3.24). This result confirms that knocking out AtBG_PPAP slightly restores the accumulation of callose at the PD in both WT and gsl8 hypocotyls, similar to what was observed in primary roots (Fig. 3.22). Moreover, PDLP5 overexpression induces callose deposition at the PD in WT, but, surprisingly, not in the gsl8 mutant.

3.7 Identification of GSL8 interacting partners

It has been predicted that GSL might be part of a complex called CalS complex (see section 1.2.4). At least four potential candidates have been suggested as components of this complex including at least one of the GSLs, UGT1, ROP and SuSy (Andrawis et al., 1993; Shin and Brown, 1999; Hong et al., 2001a; Hong et al., 2001b).

Additionally, it has been indicated that a balance between β-1,3-glucan synthase and glucanase activities is required to achieve a dynamic modulation of callose deposition at the PD, and consequently regulate the SEL of the PD in response to exogenous and endogenous stimuli (Fig. 1.5) (Yadav et al., 2014). PDLPs have also been characterized as PD-localized proteins and, in theory, they can interact with other proteins that are similarly-
Figure 3.23 Phenotype of PDLP5-OE seedlings and PDLP5 subcellular localization. (A-C) Morphological phenotypes of wild-type Col-0 (A), PDLP5-OE (B) and essp8 PDLP5-OE (C) two-week-old seedlings. PDLP5-OE seedlings show chlorosis compared to the WT. essp8 seedlings overexpressing PDLP5 display a similar phenotype as essp8. (D-F) Subcellular localization of PDLP5 in PDLP5-OE (D) and essp8 PDLP5-OE (E and F) five-day-old primary roots. In the WT background, PDLP5 is localized at the cell membranes in a dotted pattern. In the essp8 background, the PDLP5-GFP signal can also be detected inside the cell periphery (white arrowhead). (G-I) Visualization of callose deposition at the PD (white arrowheads) using aniline blue staining in WT Col-0 (G), PDLP5-OE (H) and essp8 PDLP5-OE (I). Scale bars: A-C = 1 mm, D-F = 20 µm, G-I = 50 µm.
**Figure 3.24** Measurement of symplastic movement in various genetic and transgenic backgrounds.

Cell-to-cell communication was quantified in hypocotyls of seven-day-old seedlings using a 10 kDa fluorescent probe (fluorescein) in corresponding genetic backgrounds as indicated. In all cases, values represent the mean ± S.E. (n = 5) and dotted lines indicate which samples were pairwise compared. Significant differences at $P < 0.01$ using Student’s t-Test are shown with **.
localized (Thomas et al., 2008). The receptor-like characteristics of PDLPs suggests that they might be involved in the regulation of symplastic trafficking. Additionally, earlier reports demonstrated that GSL8 is required for cytokinesis completion and proper callose deposition during cell plate formation (see section 3.4) (Chen et al., 2009; Thiele et al., 2009). SCD1 has been known to function in membrane trafficking during cytokinesis and cell expansion and, together with SCD2, is associated with clathrin-mediated membrane transport and plasma membrane endocytosis (McMichael et al., 2013). SCD1 was chosen as one of the candidate to investigate its possible interaction with GSL8 at cell plate and/or plasma membrane.

To determine the components of the callose synthase complex, firstly a hypothetical interactome was generated for GSL8 using a web-based tool (Franceschini et al., 2013) (Fig. 3.25). By combining the results from the hypothetical interactome and previous studies, six candidates, SUCROSE SYNTHASE 1 (SUS1), UDP-GLYCOSYL TRANSFERASE (UDPG), ROP4, PDLP5, AtBG_PPAP and SCD1, were chosen to investigate their physical interaction with GSL8 in vivo.

FRET was used to test these protein-protein interactions. The acceptor photobleaching approach (see section 2.9) was used to quantify FRET efficiency. FRET analysis showed an in vivo interaction between GSL8 and SUS1, AtBG_PPAP, PDLP5 and SCD1. However, no direct interaction could be detected between GSL8 and UDPG or ROP4 (Fig. 3.26). Testing the swapped fluorophores, in which GSL8 was used as the donor, generated similar results (data not shown), confirming the observed molecular interactions.

These results suggest that, similar to cellulose synthase complex, SuSy is likely to provide the UDP-Glc for callose synthase, e.g. GSL8, confirmed by identification of their interaction. The interaction of PD-localized β-1,3- glucanase (AtBG_PPAP) with GSL8 provides evidence that the balance between the activity of enzymes synthesizing and degrading callose might occur through their direct interaction. Based on our results as well as the previous prediction (De Storme and Geelen, 2014), induction of callose deposition at the PD by PDLP5 is suggested to be mediated by GSL8 as shown by their interaction. An earlier study had predicted the possible interaction of SCD1 and callose synthase during cell plate formation (Hong et al., 2001a) as was confirmed here.
Figure 3.25 A hypothetical interactome network for GSL8.

Potential interactors of GSL8 were identified using a web-based tool (Franceschini et al., 2013). Different line colors represent the type of evidence used to identify the association. Functional partners of GSL8 were identified based on co-expression (grey), experimental data (pink), database (blue), text mining (green) and sequence homology (purple) (Franceschini et al., 2013).
Figure 3.26 GSL8 interacts with sucrose synthase, AtBG_PPAP, PDLP5 and SCD1. The acceptor photobleaching FRET showed an interaction between GSL8 and SUS1, PD-localized callose degrading enzyme (AtBG_PPAP), PDLP5 and SCD1. According to the FRET results, no direct interaction could be identified between GSL8 and UDPG, and ROP4. Free CFP was used as the negative control. The data used for FRET efficiency calculation and statistical analyses were obtained from at least three independent experiments and three biological replicates for each experiment. In all cases values reported are the mean ± S.E., n = 9.
3.7.1 **GSL8 cytoplasmic domain is required for its interaction with SUS1, AtBG_PPAP, PDLP5 and SCD1**

The alignment of GSLs’ amino acid sequence revealed that the conserved residues mostly belong to the cytoplasmic domain with the longest conserved region located on the C-terminal end of the cytoplasmic loop in GSL8 (Appendix VII, Fig. 3.12). Based on the GSLs’ alignment, it was hypothesized that the cytoplasmic loop of GSL8 acts as its catalytic site and is required for the interaction with its identified partners. Therefore, truncation of GSL8 in *essp8*, leading to the absence of the cytoplasmic loop, should negatively affect GSL8 function. To assess this hypothesis, YFP and CFP fusions were made with the truncated version of GSL8 to use for interaction analysis. The interaction of truncated GSL8 with SUS1, AtBG_PPAP, PDLP5 and SCD1, which was proved with full-length GSL8, was investigated using FRET. For all four interactions, FRET quantification revealed significant decrease compared to the full length version (Fig. 3.27). Thus, it can be concluded that the GSL8 cytoplasmic loop is indeed required for its interaction with its partners and, therefore, for its activity.

3.7.2 **GSL10 might be part of the callose synthase complex**

GSL8 and GSL10 are the most closely-related members of the GSL family, at both their genomic and amino acid sequence levels, and clustered into the same subfamily in the phylogenetic tree of the Arabidopsis GSL family (Fig. 3.11). Additionally, their loss-of-function mutants show similar phenotypes during microspore mitosis and sporophyte development (Töller et al., 2008). The hypothetical interactome for GSL8 also suggested high likelihood of its interaction with GSL10 (Fig. 3.25). These results led to the speculation that GSL8 and GSL10 form a heterodimeric complex, in which the absence of one member disrupts the complex functionality. This is similar to what has been shown for cellulose synthase (CESA) complexes which consist of three different CESA proteins (Taylor et al., 2000; Gardiner et al., 2003).

A previous study reported dwarf phenotypes for both *gsl8* and *gsl10* knockdown lines, generated using dsRNAi approach, with *gsl10* exhibiting more severe dwarfism (Töller et al., 2008). Therefore, it was hypothesized that *gsl8 gsl10* double mutants show similar
Figure 3.27 The GSL8 cytoplasmic loop is required for its interaction with SUS1, AtBG_PPAP, PDLP5 and SCD1.

Using a truncated GSL8 as the acceptor in FRET quantification for GSL8 showed significant reduction of FRET efficiency for all four interactions with SUS1, AtBG_PPAP, PDLP5 and SCD1 compared to full-length GSL8. The data used for FRET efficiency calculation and statistical analysis were obtained from at least three independent experiments and three biological replicates for each experiment. In all cases values reported are the mean ± S.E., n = 9. The double asterisks denote significant differences using Student’s t-Test, P < 0.01.
phenotype to that of gsl8 or gsl10 single mutants if GSL8 and GSL10 indeed form a heterodimeric complex.

Due to the essential role of GSL10 in gametophytic development, no homozygous gsl10 mutants could be recovered after screening several T-DNA insertion lines for GSL10 (Appendix I). Therefore, gsl8 gsl10 double mutant was generated using an artificial miRNA under the control of an estradiol-inducible promoter (see section 2.6.4). XVE::aMIRGSL8/GSL10 transgenic seeds were germinated and induced on MS media containing 100 µM β-esteradiol or mock-treated on MS media containing DMSO. The mock treated fourteen-day-old XVE::aMIRGSL8/GSL10 transgenic seedlings did not show any obvious defects compared to the WT Col-0 (Fig. 3.28A-B), whereas the transgenic seedlings treated with β-esteradiol phenocopied the phenotype of gsl8-1 mutant seedlings. Similar to gl8-1, XVE::aMIRGSL8/GSL10 transgenic seedlings show dwarfism and develop abnormally-shaped true leaves. Additionally, the primary roots and root hairs of XVE::aMIRGSL8/GSL10 seedlings are shorter, thicker, and the root tips are swollen compared to the wild-type, but similar to those of gsl8-1 (Fig. 3.28C-D).

This result suggested that GSL8 and GSL10 are unlikely to be functionally-redundant, as their single mutants have strong phenotypes, although it is possible that they become functional by forming a complex. Hence, the XVE::aMIRGSL8/GSL10 seedlings do not exhibit more severe defects compared to gsl8 single mutants. Further investigation is required to determine whether GSL8 and GSL10 physically interact in vivo.
Figure 3.28 Morphological phenotype of XVE::aMIRGSL8/GSL10 seedling. Comparing the morphological phenotypes of two-week-old WT Col-0 (A), mock-treated (B), XVE::aMIRGSL8/GSL10 transgenic (C) and gsl8-1 (D) seedlings. XVE::aMIRGSL8/GSL10 transgenic seedlings phenocopy the gsl8-1 single mutant developmental defects: dwarf seedlings with short and thick roots (white arrowhead), and abnormally-shaped true leaves (red arrowhead). Scale bars = 1 mm
CHAPTER 4
4 DISCUSSION

4.1 essp8 is a new allele of GSL8

In this study, an Arabidopsis mutant named essp8, initially identified through an unrelated genetic screen (see section 1.8), was discovered to have an interesting phenotype. essp8 mutant forms tiny seedlings, having short and thick roots and hypocotyls. The cotyledons and true leaves are thicker and undulated in essp8 seedlings compared to the WT. In addition, the essp8 mutation can ectopically induce somatic embryo formation with incomplete penetrance. The first evidence for the biological role of essp8 was discovered when essp8 was identified as a novel allele of Arabidopsis GSL8. GSL8 has been characterized as a callose synthase. Callose plays important roles in cell division, growth and differentiation in plants (Piršelová and Matušíková, 2013), and accounts for up to 80% of the plant cell walls’ dry mass (Xie and Hong, 2011). Hence, this study was focused on characterization of essp8 mutant.

The observed essp8 seedling phenotype is similar to the defects previously reported for the other GSL8 alleles, such as showing dwarfism, short roots with defects in root tissue patterning (Chen et al., 2009; Thiele et al., 2009; Guseman et al., 2010), swollen, branched root hairs (Guseman et al., 2010), and disorganized epidermal cells in cotyledon and true leaves (Chen et al., 2009). However the ectopic somatic embryo phenotype had not been documented for gsl8 mutants. An elevation of cell proliferation in the root cap collumella, hypocotyl cortex, endodermis and phloem (Chen et al., 2009), stomatal-lineage cells (Guseman et al., 2010), and cotyledon and first leaves (De Storme et al., 2013) was mentioned in previously characterized alleles of GSL8. My different observation might be due to the unique mutation site in essp8 leading to the truncation of GSL8. Alternatively, it cannot be excluded that it might be simply caused by slightly different growth conditions such as difference in sucrose concentration in the growth media compared to what were used in other studies (3% vs. 1%). Dependence of aerial and root morphological phenotype, and cell expansion on external sucrose level has been previously reported (Benfey et al., 1993; Roldán et al., 1999; Caño-Delgado et al., 2000; Takahashi et al., 2003). This study also showed the dependence of somatic embryo phenotype induction on the sucrose concentration. As such, essp8 seedlings develop
somatic embryos only on MS media containing 3% sucrose (Fig. 3.3), whereas, no somatic embryo could be identified when the sucrose content was decreased to 1.5% (data not shown). This is the first report on induction of somatic embryo formation and cell identity change in a gsl8 mutant. The somatic embryo phenotype of essp8 mutant provides new insight into GSL8 requirement during early seedling development in Arabidopsis.

4.1.1 gsl8 mutation causes embryo defects and seedling lethality

Poor germination was observed, regardless of sucrose concentration, in all the GSL8 mutants studied, including gsl8-1, gsl8-2, gsl8-3, gsl8-4 and essp8. Germination failure in about 5% of the homozygous essp8 seeds resulted in a segregation ratio of approximately 20%, instead of 25% (Table 3.1), the ratio expected in the case of Mendelian segregation for a recessive mutation at a single locus. The morphological analysis of the siliques from a heterozygous parent showed that homozygous gsl8 seeds are reduced in size compared to the WT and carry defective embryos (Fig. 3.1E-F), which is consistent with previous reports (Chen et al., 2009; Thiele et al., 2009).

The lethality for the gsl8 knockout lines has already been documented (Töller et al., 2008; Chen et al., 2009; Thiele et al., 2009; Guseman et al., 2010). The lethal phenotype of essp8 mutant seedlings excludes the possibility of GSL8 functional redundancy with the other eleven identified GSLs in Arabidopsis, at least at the seedling stage. Additionally, it indicates that GSL8 plays a specific and substantial role in callose biosynthesis during plant growth and development. High correlation of GSL8 and GSL10 expression profiles and their similar mutant phenotypes during microspore mitosis and sporophyte development have raised the question of whether they are functionally-related, or have overlapping roles (Töller et al., 2008). It still needs to be further investigated if GSL8 and GSL10 can form a heteromeric callose synthase complex, similar to what have been shown for cellulose synthase complexes (Taylor et al., 2000; Gardiner et al., 2003). The existence of such a complex can potentially explain their similar mutant phenotypes, caused by disruption of the complex activity in the absence of one component (see section 4.5.4).
4.2 *gsl8* phenotypic defects are partially caused by cytokinesis impairment

Endopolyploidization, defined as the cell-specific nuclear DNA content multiplication, in both plants and animals is either the result of endomitosis or endoreduplication during mitotic cell cycle processes (Joubès and Chevalier, 2000). In endoreduplication, due to a complete absence of mitotic chromosomes condensation and their subsequent division, while the total number of the chromosomes won’t be affected, polytenal chromosomes are generated (Edgar and Orr-Weaver, 2001; Lee et al., 2009). In endomitosis, in contrast to endoreduplication, cells enter mitosis but do not complete the process. Depending on the stage when the M-phase is aborted, division of the duplicated chromosomes and cytokinesis might be missing, resulting in the generation of cells that will have doubled absolute number of chromosomes (D’Amato, 1984).

Although callose biosynthesis occurs in different species, accumulation of callose at the cell plate during cell division is plant-specific, and has been confirmed for all plant taxa (Scherp et al., 2001). Callose is known as a major component of cell plate and cell wall and, therefore, is required for completing plant cytokinesis and proper cell wall formation (Verma, 2001; Chen and Kim, 2009; Vatén et al., 2011; Piršelová and Matušíková, 2013). It has been shown that loss-of-function mutations in *GSL8* cause defects in cell plate and cell wall formation (Töller et al., 2008; Chen et al., 2009; Thiele et al., 2009; Guseman et al., 2010; De Storme et al., 2013). Thiele et al. (2009) for the first time reported severe cell division defects and a significant decrease in callose deposition at the cell plates in a *GSL8* knockdown line. This result provided the evidence supporting the important role of *GSL8* in callose biosynthesis during cytokinesis. Additionally, defects in the cell walls accompanied with formation of endomitotic cells were detected in flower tissues of *et2*, a weak allele of *GSL8* (De Storme et al., 2013). Taken together, callose biosynthesis by *GSL8* plays a substantial role during cell plate formation and is thus required for maintaining the basic ploidy level, especially in reproductive tissues. My findings are in agreement with the previous studies and reconfirm the cytokinesis defects in the newly identified allele of *GSL8*, *essp8*. The *essp8* mutation causes reduction of callose deposited at the cell plates in actively dividing cells in the RAM. Determination of the ploidy level in the root cells of three different *gsl8* mutants, *essp8*, *gsl8-1* and
gsll-2 revealed that gsll mutation induces ectopic polyploidization and endomitosis, both in the meristematic tissue and elongating cells of the root (Fig. 3.15 and 3.16). This result, for the first time, provides the evidence that cytokinesis defects in gsll mutants are beyond the reproductive tissues and affect both somatic and reproductive cells.

4.2.1 Cytokinesis defects might cause lethality of essp8 seedlings

The cause(s) of gsll knockout mutants’ lethality is still under debate. Loss of proper chromosome condensation and segregation during successive cell divisions has been suggested as one of the potential reasons leading to growth arrest (De Storme et al., 2013). Segregation of the replicated chromosomes becomes too complicated in polyploid or endomitotic nuclei as they go through consecutive cell divisions (Sugimoto-Shirasu and Roberts, 2003). Therefore, it is expected that with progression of gsll seedling development, the number of polyploid and/or endomitotic cells and the ploidy level increases. Comparing essp8 seedlings at different ages confirmed that more cells display cytokinesis defects as the number of polyploid and/or endomitotic cells significantly increases in older seedlings (Fig. 3.17). It is plausible that accumulative polyploidization caused by defects in cell plate formation induces a premature arrest of cell division in proliferating tissues and, consequently, cell death. Death through mitotic catastrophe and polyploidization has already been reported in different species (Doelling et al., 2001; Tzafrir et al., 2002; Castedo et al., 2004; McCall, 2004; Vakifahmetoglu et al., 2008; Pritchett et al., 2009).

4.2.2 Cytokinesis-defective mutants do not exhibit severe tissue impairments

Ectopic polyploidization is known as a general characteristic of cytokinesis-defective mutants and has been detected in different organisms and cell types (Hatzfeld and Buttin, 1975; Thompson and Lindl, 1976; Karess et al., 1991; Castrillon and Wasserman, 1994; Neufeld and Rubin, 1994; Liu et al., 1997; Spielman et al., 1997; Lordier et al., 2008; Pampalona et al., 2012; Serres et al., 2012; De Storme and Geelen, 2013). Through forward genetic screens, several cytokinesis-defective mutants have been isolated. Comparing the phenotypic defects of three gsll alleles to five different cytokinesis-defective mutants including hinkel, scd1, knolle, keule and korrigan showed that
dwarfism is shared among all the analyzed mutants. As was expected, all cytokinesis-defective mutants induce ectopic polyploidization in the root somatic cells similar to that observed for gsl8 mutants. However; root tissue patterning and root-hair morphogenesis do not exhibit severe impairments as seen in gsl8 (Fig. 3.14 and 3.15). For example, the keule mutant roots are stunted (Söllner et al., 2002; Thiele et al., 2009), but bloated cells and branched swollen root hairs, characteristic for essp8, could not be detected in keule seedlings. Additionally, none of the cytokinesis-defective mutants showed somatic embryo formation.

With the exception of KORRIGAN, the remaining cytokinesis-defective mutants analyzed in this study are required for vesicles transport and fusion, known as the first step of cell-plate formation (Samuels et al., 1995; Lukowitz et al., 1996; Waizenegger et al., 2000; Strompen et al., 2002). Callose deposition, however, mainly occurs during the second step of cell-plate formation where the cell wall is assembled within the network generated in the first step as a result of vesicle fusion (Thiele et al., 2009). Therefore, part of the phenotypic differences might be caused by the disparate timing of their involvement during cytokinesis completion. KORRIGAN encodes a membrane-bound endo-1,4-β-glucanase that plays a role during the second step (Sato et al., 2001). Surprisingly, in spite of their related functions during cytokinesis, GSL8 and KORRIGAN mutants display different phenotypes. My findings suggest that essp8, similar to the other reported GSL8 alleles (Chen et al., 2009; Thiele et al., 2009; De Storme et al., 2013), is a cytokinesis-defective mutant, as indicated by its lack of callose deposition at the cell plates and ectopic induction of multi-nucleate and endomitotic somatic cells. Nevertheless, essp8 phenotypic defects and seedling lethality are likely to be caused only 'partially' by cytokinesis impairments.

It was previously proposed that GSL6 is a cell plate-specific callose synthase (see section 1.2.2). In tobacco BY-2 cells, GFP-GSL6 fusion proteins are localized to the cell plate when expressed under the control of the CaMV35S promoter (Hong et al., 2001a; Hong et al., 2001b). Interestingly, gsl6 knockout lines do not show any phenotypic and/or cytokinesis defects (Dong et al., 2008; Chen et al., 2009). Therefore, considering the severity of cytokinesis defects as well as the observed lethality in different gsl8 mutants,
tested in this study, it is tempting to propose that GSL8 is the callose synthase essential for depositing callose at the cell plate to complete cytokinesis.

4.3 Cell-to-cell communication is relaxed in essp8 hypocotyls

Intercellular signaling is required for regulation of developmental programs in any multicellular organism. In plants, PD channels interconnect the cells and provide the path for movement of nutrients and regulatory molecules such as mobile transcription factors (Rinne and van der Schoot, 1998; Koizumi et al., 2011; Xu et al., 2011). Callose deposition at the neck of the PD plays a critical role in regulating cell-to-cell communication in plants (Vatén et al., 2011). Symplastic signaling is a dynamic process and is achieved through a balance between the activity of callose synthases and β-1,3-glucanases (Levy et al., 2007). However, it is still not clear how callose synthases regulate PD and what other molecular components are required for this regulation.

Expression analysis of GSL8 indicated that GSL8 is highly expressed in most organs during plant development with the maximum transcript level in the vasculature and actively dividing cells (Schmid et al., 2005; Chen et al., 2009). In the vasculature, callose is mostly deposited at the PD to regulate its SEL. Therefore, GSL8 was suggested to be associated with PD regulation. In agreement with the predicted role of GSL8 in PD regulation, a mutant allele of GSL8, called chorus, showed PD defects as indicated by an increase in passive diffusion of CFP and a trimeric GFP (3×GFP) molecules in leaf epidermis (Guseman et al., 2010). In agreement with Guseman at al. (2010), the histochemical assay to visualize the callose deposited at the PD showed that, in all gsl8 mutants, almost no callose is accumulated at the PD in the elongation zone of the root, in the root hairs and vasculature tissue compared to the wild-type Col-0 (Fig. 3.13E-H). Furthermore, the cell-to-cell diffusion assay using fluorescent probes demonstrated that the essp8 mutation results in an increase in symplastic movement in hypocotyls, likely due to lack of callose accumulation at the PD, which consequently boosts the passive diffusion (Fig. 3.18). Therefore, not only my finding is consistent with PD defects previously documented in chorus epidermal cells by Guseman et al. (2010), but also it indicates that PD defects in gsl8 mutants occur in different somatic tissues. Hence, it can
be concluded that GSL8 is required for callose deposition at the PD and regulation of their size exclusion limit.

Two other members of the Arabidopsis GSL family, GSL7 and GSL12, were recently shown to be involved in callose biosynthesis at the PD (Barratt et al., 2011; Vatén et al., 2011; Xie et al., 2011). Compared to GSL12, GSL7 callose biosynthesis activity is more tissue-specific. GSL7 is expressed in the vasculature system and is required only for callose deposition at the phloem PD and sieve plates. Vatén et al. (2011) suggested a role for GSL12 in PD regulation using an inducible expression system for GSL12 gain-of-function mutants. Although, a GSL12 gain-of-function mutant seedlings develop shorter roots compared to the WT, the defect is not as severe as those of essp8 (Vatén et al., 2011; Yadav et al., 2014). The lethality of essp8 single mutant rules out the possibility that GSL8 and GSL12 are functionally redundant during early seedling development and suggests that they are likely to be independently required for callose biosynthesis at the PD with GSL8 playing a more critical role.

4.3.1 **GSL8 is required for highly-regulated trafficking of SHR**

To gain a better understanding of the effect of essp8 mutation on PD aperture and trafficking of various mobile factors, SHR (see section 1.5.2) was chosen as a candidate for NCATFs, and its symplastic movements in essp8 roots was investigated. SHR is specifically expressed in the stele cells and the SHR protein moves from stele into the neighboring cells including the QC, the CEIs, the CEIDs and the endodermis (Nakajima et al., 2001). In the endodermis, SHR transcriptionally activates several target genes including SCR. Physical interaction of SHR and SCR suggests that a transcription activation complex might be formed by the two proteins (Nakajima et al., 2001; Levesque et al., 2006; Cui et al., 2007).

Vatén et al. (2011) suggested that SHR movement is likely to be PD-mediated as callose induction, in either the endodermis or the stele, could impair its trafficking (Vatén et al., 2011). Disruption of SHR movement in essp8 root meristematic zone suggests dysregulation of SHR signaling pathway. This finding, for the first time, provides evidence that essp8 mutation indeed alters the movement of SHR protein as its trafficking
increases by an extra cell layer. This increase could be detected as early as in five-day-old *essp8* roots. In *essp8*, SHR ectopically moves to the cortex, the neighboring cells to endodermis (Fig. 3.19B). In wild-type seedlings, SHR is localized into both nucleus and cytoplasm of stele cells, but only to the nucleus in the endodermis (Fig. 3.19A) where it activates *SCR* as a transcription factor (Cui et al., 2007). Contrary to the WT, in *essp8* seedlings, SHR was localized into both, nucleus and cytoplasm, in stele, endodermis and cortex cells (Fig. 3.19B). Previous studies have shown that restricted movement of SHR for only one cell layer and its nuclear localization in the endodermis are critical regulatory mechanisms for endodermis differentiation (Nakajima et al., 2001), and are evolutionarily conserved (Cui et al., 2007). Reduction of SHR nuclear localization and its movement outside of the endodermis induces cell divisions and an increase in the number of cell layers, where the cells ectopically exhibit endodermal characteristics (Cui et al., 2007; Welch et al., 2007; Miyashima and Nakajima, 2011).

SHR plays a substantial role during embryonic root development by regulating radial patterning of the Arabidopsis root (Nakajima et al., 2001). Transport of SHR from the stele into the endodermis acts as an initiator of cell communication network in the meristematic zone of the root. SHR is required for cell division and endodermis differentiation (Benfey et al., 1993; Helariutta et al., 2000). Highly-regulated movement of SHR and its well-defined subcellular localization are both crucial for ground tissue formation. Furthermore, it has been previously shown that SHR abundance changes dynamically during root development, and its dose regulates MC formation. An increase in SHR level prevents MC formation, whereas its abundance at intermediate level promotes MC formation (Koizumi et al., 2012). Additionally, overexpression of *SHR* using *CaMV35S* promoter induces disorganized cell divisions in the root meristem (Helariutta et al., 2000; Nakajima et al., 2001). Similarly, ectopic expression of *SCR* in the supernumerary cell layers leads to development of extra endodermal layers (Cui et al., 2007). Collectively dysregulation of SHR symplastic movement in *gsl8* mutants suggests that GSL8 is required for careful regulation of SHR trafficking via PD. Therefore, it is tempting to speculate that *essp8* root defects such as increased number of cell layers and cell identity changes are, at least partially, caused by impairment of SHR cell-to-cell movement caused by loss of GSL8 and disruption of PD aperture. These results suggest a
new role for GSL8 as a critical factor required for regulation of cell communication network during early seedling development in Arabidopsis.

4.3.2 Loss of GSL8 dysregulates symplastic movement of microR165/6

Transcription activation of three *MICRORNA165/6* genes, *MIR165A*, *MIR166A* and *MIR166B* is SHR-mediated in the QC, CEIs, CEIDs and endodermis (Carlsbecker et al., 2010; Miyashima et al., 2011). SHR directly binds to the 5’ upstream regions of *MIR165A* and *MIR166B* to activate their transcription (Carlsbecker et al., 2010). After activation, the mature miR165/6 will move from endodermis to stele, and suppress the *HD-ZIP III* family genes, such as *PHB*, by targeting their transcripts (McConnell et al., 2001; Prigge et al., 2005; Hirakawa et al., 2011). Suppression of the *HD-ZIPIII* genes by miR165/6 plays important roles in establishing apical-basal polarity during embryogenesis, patterning of leaf primordia and shoot vascular organization (McConnell et al., 2001; Emery et al., 2003; Smith and Long, 2010). However, it is not yet clear whether miR165/6 acts as a non-cell-autonomous factor during the other developmental events, or if it is root-specific.

Similar to SHR, symplastic movement of miR165/6 has been shown to be PD-mediated (Vatén et al., 2011). The symplastic movement of mature miR165/6 was investigated in *essp8* roots to confirm whether PD defects and the increase in their SEL affect miR165/6 signaling. The analysis revealed that the movement of mature miR165/6 is impaired in *essp8* roots (Fig. 3.20). Even though the difference in miR165/6 activity between wild type and *essp8* in five-day-old seedling roots was minor, the ectopic activity of miR165/6 in the outer cell layers and root collumella cells increases markedly in ten-day-old roots as the root tissue patterning develops a more severe defect. This is the first report on the alteration of miR165/6 intercellular movement in *gsl8* mutants. This new finding suggests that GSL8 is required for regulation of miR165/6 PD-mediated trafficking.

The mRNAs of five *HD-ZIPIII* genes, including *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA/INTERFASCICULAR FIBERLESS1* (*REV/IFL1*), *CORONA/INCURVATA4* (*CNA/ICU4*) and *ATHB8*, are targeted by miR165/6 (Baima et al., 1995; Talbert et al., 1995; McConnell et al., 2001; Green et al., 2005; Prigge et al., 2005;
Ochando et al., 2006). Although single mutants for any of these five genes do not display distinguishable defects in their root tissue patterning, quadruple mutants for the *HD-ZIPIII* genes, as well as overexpression lines for miR165 develop excessive cell layers in their vasculature tissue (Carlsbecker et al., 2010). The dose of miR165/6 in the ground tissue precisely regulates the PHB distribution across the stele, known to be essential for xylem differentiation in vasculature tissue (Miyashima et al., 2011). Interestingly, miR165/6 and HD-ZIPIII have also been found to be involved in regulation of xylem secondary cell wall (SCW) formation (Du and Wang, 2015). Alternatively, miR165/6 seems to have a bidirectional movement pattern, moving both, to the inside and outside, of the endodermis (Carlsbecker et al., 2010). Therefore, miR165/6-mediated suppression of the *HD-ZIPIII* genes in the endodermis and stele is not only restricted to xylem cell specification, but also plays a broader role in cell layers differentiation in Arabidopsis roots (Miyashima et al., 2011). The defects in meristematic root tissue patterning in *essp8* mutants are similar to the phenotype of miR165/6 overexpression lines and quadruple mutants of *HD-ZIPIII* genes, particularly the extra cell layers observed in the root and vasculature tissue (Carlsbecker et al., 2010). Therefore, it can be suggested that ectopic miR165/6 activity in the outer cell layers in the root meristem might be, at least partially, responsible for the *essp8* root phenotype and loss of vasculature tissue in ten-day-old seedlings.

Significant decrease of miR165/6 levels in both *shr* and *scr* mutants have suggested that the SHR/SCR transcription complex is likely to regulate *MIR165/6* expression (Carlsbecker et al., 2010; Miyashima et al., 2011). Therefore, it still needs to be further investigated whether elevated miR165/6 activity in the outer cell layers in *essp8* is a direct result of an increase in its bidirectional PD-mediated symplastic movement from endodermis to stele, cortex, epidermis and collumella cells, or the ectopic presence of SHR in the outer cell layers up-regulates miR165/6, or it is caused by both.

### 4.4 PDLP5 function is likely to be GSL8-dependent

PDLP isoforms were first identified in the cell wall proteome of Arabidopsis (Table 1.1) (Thomas et al., 2008). PDLPs are small proteins with a predicted size of 30 to 35 kDa. They contain a very short tail at the C-terminus, two conserved Cys-rich repeats at the N-
terminus and a connecting transmembrane domain (TMD) (see section 1.4.1). Thomas et al. (2008) showed that all PDLPs localize to the cell periphery with punctate structures which represent PD. There are reports indicating that overexpression of PDLP1 and PDLP5 leads to the reduction in PD permeability and, therefore, improves plants’ resistance against pathogens, and concomitant elevation of callose deposition at the PD (Lee et al., 2008; Thomas et al., 2008; Lee et al., 2011; Wang et al., 2013). It is still unknown how PDLPs induce callose deposition at the PD and control symplastic trafficking in response to both internal and external signals. Contrary to PDLPs positive regulation of callose deposition at the PD, Amari et al. (2010) found that PDLPs might act as endogenous factors required for virus infection and movement of the virus MP. It was found that in a pdlp triple mutant, the virus association with the PD and its MP movement is inhibited (Amari et al., 2010).

To investigate whether the gsl8 phenotypic defects can be partially rescued by induction of callose deposition at the PD, PDL5 was overexpressed in a gsl8 genetic background. Seedlings overexpressing PDL5 look slightly yellowish, and their adult plants show evident growth inhibition and chlorosis compared to the WT (Fig. 3.23A-B). This result is consistent with previous reports on the phenotype of PDL5 overexpression lines (Lee et al., 2011; Carella et al., 2015). Similar to the other members of the PDLP family (Thomas et al., 2008; Lee et al., 2011), the localization analysis in the root showed that PDLP5 is localized at the cell wall as punctate structures, correspond to PD, in the WT and gsl8 five-day-old seedlings (Fig. 3.23D-E). Interestingly, serial z-section images revealed that in essp8 seedlings overexpressing PDL5, the punctate localization pattern is missing in some of the cells. Instead, PDL5-GFP signal was detected in the cytoplasm (Fig. 3.23C).

Dysregulation of PD aperture in gsl8 mutant seedlings was previously observed as it affects the symplastic movement of non-cell autonomous factors including SHR and miR165/6 (see sections 4.3.1 and 4.3.2). Mislocalization of PDLP5 to cytoplasm in five-day-old gsl8 seedlings raised the question whether PDLP5 localization at the PD is GSL8-dependent, or this occurs only as an indirect effect of cell wall and PD defects in gsl8 mutants.
Lee et al. (2011) showed that overexpression of *PDLP5* induces callose accumulation at the PD for up to fourfold higher than the wild type. They proposed that PDLP5 controls PD permeability through modulation of callose accumulation at the PD. In agreement with their finding, WT seedlings overexpressing *PDLP5* display an increase in callose deposited at the PD compared to the wild type. Furthermore, over accumulation of callose at the PD in *PDLP5-OE* lines indeed affects the SEL of PD and restricts passive diffusion through PD (Fig. 3.24). This finding supports the notion of Lee et al. (2011) that PDLP5 adjusts callose accumulation at the PD to control PD permeability.

In contrast to the *PDLP5-OE* data in wild type, surprisingly, overexpression of *PDLP5* in a *gsl8* mutant background did not alter callose accumulation at the PD compared to the *gsl8* loss-of-function mutants. Moreover, no significant difference could be detected in intercellular movement of fluorescein in the hypocotyls of *gsl8 PDLP5-OE* seedlings (Fig. 3.24). Taken together, these results explain why overexpression of *PDLP5* in *gsl8* background cannot rescue their phenotypic defects as persistent lack of callose at the PD in *gsl8 PDLP5-OE* lines does not lead to discernible restoration of the PD size exclusion limit. Therefore, it can be concluded that loss of GSL8 might negatively affect PDLP5 function and subcellular localization.

De Storme and Geelen (2014) proposed that negative regulation of PD by PDLPs might occur through their interaction with enzymes synthesizing callose such as GSLs; however, no evidence was provided to support this hypothesis. This study provides novel insight into a putative cross talk between GSL8 and PDLP5, and the significance of this cross talk in PD regulation. Based on PDLP5 mislocalization and absence of callose induction in *gsl8 PDLP5-OE* seedlings, it can be argued that PDLP5 is likely to require GSL8 for its localization at the PD and this might happen through their direct interaction. This assumption needs to be further confirmed by testing the possibility of PDLP5 and GSL8 physical interaction, and if truncation of GSL8, in case of *essp8*, affects this putative interaction (see sections 4.5.2 and 4.5.5).
4.5 **GSL8 forms a complex to synthesize callose and regulate its deposition**

Callose biosynthesis and its deposition need to be highly-regulated. Hence it has been proposed that GSLs, e.g. GSL8, are integrated into an extremely specialized protein complex, hypothetically named CalS complex, to carry out callose synthesis.

4.5.1 **SUCROSE SYNTHASE 1, a provider of UDP-Glc to GSL8**

Callose is synthesized from hundreds of glucose residues linked by β-1,3- glycosidic bridges (Piršelová and Matušíková, 2013). Thus, glucose molecules need to be provided to GSLs, where they are used by GSLs as precursors to synthesize β-1,3-glucan polymers. SuSy, known as a sugar metabolic enzyme involved in sucrose synthesis and degradation, can potentially act as the glucose provider for GSLs. It has been previously shown that in cellulose synthase complexes, SuSy is in charge of UDP-Glc delivery to the enzyme (Baroja-Fernández et al., 2012).

The FRET analysis (see section 3.7) revealed a physical interaction between GSL8 and SUS1 (Fig. 3.26). This result suggests that, similar to the case of the cellulose complex, SuSy is likely to be incorporated into the callose synthase complex to channel UDP-Glc into glucan synthesis. SUS1 is traditionally predicted to be localized into cytosol (Hooper et al., 2014). However, several studies have suggested that, depending on the metabolic environment, SUCROSE SYNTHASE can adjust its cellular localization, and interact with membranes, organelles and cytoskeletal actin (Winter and Huber, 2000; Etxeberria and Gonzalez, 2003; Subbaiah et al., 2006; Zheng et al., 2011). Several lines of evidence have highlighted posttranslational modification as an important regulatory mechanism for SUS1 association with membranes. For example, SUS1 phosphorylation at Ser-15 in *Zea mays* and Ser-11 in *Glycine max* enhances its association with the membrane (Komina et al., 2002; Hardin et al., 2004). Although the molecular mechanisms by which SUS1 can bind to the membranes are not yet known, based on this finding, it is plausible to conclude that SUS1 integration into CalS complex might occur at its membrane-associated status where it interacts with GSL8 to provide UDP-Glc monomers, required for callose biosynthesis.
4.5.2 A PD-localized β-1,3-glucanase and PDLP5 regulate PD through GSL8

AtBG_PPAP, a PD-localized enzyme degrading callose, is required for maintaining callose balance at the PD (Levy et al., 2007; Zavaliev et al., 2011). AtBG_PPAP is localized in the ER membrane and at the cell periphery where it is associated with PD (Zavaliev et al., 2013). As was previously predicted in the hypothetical interactome network for GSL8 (Fig. 3.25), results of the FRET experiment support interaction of GSL8 and AtBG_PPAP (Fig. 3.26). The physical interaction of GSL8 and PD-associated β-1,3-glucanase suggests that GSL8 is likely to be the PD-associated callose synthase required for retaining the equilibrium between callose synthesis and hydrolysis.

My results already showed that PDLP5 functionality and subcellular localization are disrupted in essp8 mutant seedlings (Fig. 3.23). This finding led me to investigate whether PDLP5 and GSL8 physically interact. The FRET result proved the interaction of GSL8 and PDLP5 (Fig. 3.26). PDLPs have their N-terminal domain exposed to the apoplast and a very short cytoplasmic domain with unknown role. Therefore, it has been proposed that the extracellular domains are likely to act as the interacting domains (Thomas et al., 2008). The FRET analysis suggests that the cytoplasmic domain of PDLP5 might be the one involved in its interaction with GSL8. Furthermore, the identified interaction between GSL8 and PDLP5 supports an earlier proposal that the induction of callose deposition at the PD by PDLP5 might happen through its interaction with callose synthase enzymes (De Storme and Geelen, 2014). Here, I have demonstrated that GSL8 is, if not the only one, the callose synthase regulating callose deposition at the PD.

Cumulatively, the physical interaction of GSL8 with two PD-localized proteins, with roles in PD regulation, provides the first evidence for an earlier prediction by Töller et al. (2008) as GSL8 might have regulatory roles, apart from its enzymatic function. This study gives new insight into the role of GSL8 in PD regulation, the mechanisms underlying the regulation of PD aperture and/or their size exclusion limit, and how they are linked to callose biosynthesis.
4.5.3 SCD1, an interacting partner of GSL8

SCD1 is required for vesicular trafficking to the equator of the dividing cell (Falbel et al., 2003), where the vesicles are fused together to form a tubulovesicular network (Söllner et al., 2002). Callose deposition converts the network into a fenestrated plate (Samuels et al., 1995; Verma, 2001).

A recent study has shown that SCD proteins, including SCD1, are involved in different membrane trafficking events required not only for cytokinesis, but also for cell expansion (McMichael et al., 2013). It is already known that the secondary PD are formed post-cytokinesis, possibly during cell expansion. However, how this happens has remained unclear (Brunkard et al., 2013). The observed interaction between GSL8 and SCD1 provides new views on the role of GSL8, both in cytokinesis and PD regulation. Therefore, it raises new questions on whether this interaction is restricted to cell plate formation and is required for callose deposition at the cell plate, and/or it is somehow linked to secondary PD formation.

4.5.4 GSL8 and GSL10 interplay might be required for proper function of callose synthase complex

The effort to clone GSL10 cDNA was unfortunately not successful, thus the interaction of GSL10 and GSL8 could not be tested. Furthermore, although five different T-DNA insertion lines for GSL10 were screened (Appendix I), no homozygous seedlings could be recovered, indicating that gsl10 mutation causes gamethophytic lethality. Hence, the seedling phenotype of a conditional gsl8 gsl10 knockdown remains as the only evidence for the hypothetical GSL8 and GSL10 interaction.

The similar phenotype of gsl8 gsl10 double mutant to that of gsl8 single mutant supports an earlier prediction made by Töller et al. (2008). They hypothesized that GSL8 and GSL10 might form a complex where both are required for the complex to be functional. The assumption that GSL8 and GSL10 form a complex, where the absence of one of them disrupts the complex function, was supported by gsl8 gsl10 morphological phenotype. Two-week-old gsl8 gsl10 seedlings’ phenotypic defects are similar to gsl8-1
by having dwarf seedlings with short roots and abnormally-shaped true leaves (Fig. 3.28). To further confirm this assumption, the physical interaction of GSL8 and GSL10 should be tested in the future. Due to requirement of both GSL8 and GSL10 in early seedling development, efforts to generate \textit{gsl8 gsl10} double mutants have not been successful. Hence, this study can be considered as the first report on GSL8 and GSL10 interplay in early seedling development in Arabidopsis.

4.5.5 **GSL8 cytoplasmic loops are required for its interaction with the partners**

GSL8 encodes a large integral membrane protein with sixteen predicated transmembrane helices and seventeen non-transmembrane loops. GSL8 comprises a large hydrophilic cytoplasmic loop in the middle (Käll et al., 2007). It has been previously suggested that the cytoplasmic loop is required for GSLs interaction with other regulatory proteins (Verma and Hong, 2001; De Storme and Geelen, 2014). The alignment of Arabidopsis GSLs’ amino acid sequence revealed that the conserved regions mostly belong to the cytoplasmic loop (Fig. 3.12). In \textit{essp8}, GSL8 is truncated at the fifth cytoplasmic loop. The findings demonstrated a loss of GSL8 interaction with SUS1, AtBG_PPAP, PDLP5 and SCD1 where an \textit{essp8} version of \textit{GSL8} cDNA was used for interaction analysis (Fig. 3.27). These data support the assumption that GSL8 cytoplasmic loop is required for its interaction with the identified partners and, possibly, its regulatory role(s). Taken together, it is predicted that truncation of GSL8 in \textit{essp8} disrupts the CalS complex at the PD and, therefore, PD regulation (Fig. 4.1).

It has been predicted that the cytoplasmic loop should contain a UDP-Glc catalytic site and a glucosyltransferase domain (Verma and Hong, 2001). Furthermore, GSLs show a high substrate specificity for UDP-Glc (Brownfield et al., 2009; Zavaliev et al., 2011). Although FRET results confirm that SUS1 may provide the UDP-Glc substrate to GSL8, no UDP-Glc binding site has been identified on the cytoplasmic loop of GSL8 yet. Therefore, further investigation should be performed to identify the specific domain(s) required for GSL8 interaction with regulatory proteins including AtBG_PPAP and PDLP5.
Figure 4.1 A proposed model for GSL8 role in PD regulation.

In wild-type plants, GSL8 negatively regulates PD SEL by forming a CalS complex and induction of callose deposition. Symplastic movement of mobile factors is blocked in the WT. Truncation of GSL8 in *essp8* mutant results in lack of a functional CalS complex at the PD and callose deposition. Absence of callose at the PD in *essp8* induces ectopic movement of mobile factors to adjacent cell layers and dysregulation of intercellular signaling.
PERSPECTIVES

The work presented in this thesis provides novel insight into the role of GLUCAN SYNTHASE-LIKE 8 during early seedling development in Arabidopsis. These new findings raise more questions for future investigation using the materials generated in this study.

Regarding the critical role of GSL8 in cytokinesis and cell plate formation, it remains to be further studied if GSL8 is solely required for callose biosynthesis at the cell plate.

Despite a clear role of callose accumulation in PD regulation, the molecular mechanism(s) underlying this regulation and how the identified players are linked to callose homeostasis have remained elusive. Identifying other interacting partners of GSL8, putative components of the callose synthase complex, would further extend our knowledge of this regulatory network. Additionally, the interaction of currently identified interactors should be reconfirmed using another protein-protein interaction method, e.g. membrane yeast two-hybrid (MYTH) system.

Vaten et al. (2011) previously reported the association of GSL12 with PD and its role in PD regulation. Therefore, it needs to be further investigated how GSL8 and GSL12 contribute to the PD regulation, and if there is any functional overlap. Additionally, the assumption of GSL8-GSL10 heterodimeric complex can be further studied by testing their subcellular localizations to find out whether they are similarly-localized.

No experimental evidence is available regarding GSL8 localization. Therefore, studying the subcellular localization of GSL8, as well as the other members of the GSL family in Arabidopsis, would provide a better understanding of their tissue-specific and subcellular localization/distribution, and elucidate if GSL8 and GSL12 are the only important regulators of plasmodesmata within the GSL family. The ability to characterize the specific roles of GSL8 and GSL12 at the PD, and understand whether they are independently required for PD regulation will also be important in this context. Furthermore, it is worth exploring the possibility of PD-associated GSLs being incorporated into one complex.
The observation that AtBG_PPAP and PDLP5 interact with GSL8, and the likelihood of GSL8 requirement for PDLP5 localization opens up the questions: which specific domain(s) in GSL8 are required for this interaction, if the interactions are only specific to GSL8 and/or other PD-associated GSLs, and whether AtBG_PPAP and PDLP5 are also components of the hypothetical PD-regulating complex?

To provide further evidence for the observed impairment of SHR and miR165/6 movement in essp8 mutant, a quantification of their target transcripts, SCR and HD-ZIP III genes, respectively, should be informative. These experiments would address the question of whether ectopic localization of these non-cell autonomous factors indeed affects the expression of their downstream targets. Finally, genome-wide expression analysis can be used to find out the global effect of gsl8 mutation on gene expression.

Many additional experiments could be listed here. The overall challenge, however, will be to gain sufficient resolution on PD cell-to-cell communication, and how it is tightly regulated in response to both internal and external cues. There is still a lot more to learn in order for us to draw a detailed picture of PD and understand the precise mechanism(s) through which GSL8 contributes to PD regulation and selective transport properties.
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APPENDICES

Appendix I List of mutant lines and primers used for genotyping

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Appendix VII Alignment of the Arabidopsis GSL family amino acid sequence

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*Appendix VII cont’d*
CURRICULUM VITAE

Behnaz Saatian

Education

Ph.D.  Western University, London, Ontario
       2010- present, Ph.D. Candidate

M.Sc.  University of Tehran, Tehran, Iran
        2002-2004

B.Sc.  Bu-Ali Sina University, Hamadan, Iran
       1997-2001

Honors and Scholarships

   Western Graduate Research Scholarship (WGRS)
   September 2010- August 2015
   Western University

   Graduate Student Travel Award
   January 2014
   Department of Biology, Western University

   Ph.D. Comprehensive Exam, Passed with Distinction
   June 2013
   Department of Biology, Western University

   Three Minutes Thesis (3MT) Competition, Finalist
   April 2013
   Western University

   Best Oral Presentation
   July 2005
   4th National Conference of Biotechnology, Kerman, Iran

Experience

   Teaching Assistant
   September 2010-present
   Western University

   Research Assistant
   September 2010-present
   Agriculture and Agri-Food Canada
Teaching Assistant Training Program (TATP) Instructor
September 2013- Present
Teaching Support Center (TSC)
Western University

Faculty of Science Lead TA
September 2013- April 2014
TSC and School of Graduate and Postdoctoral Studies (SGPS)
Western University

Director of Quality Control Laboratory
March 2009- August 2010
Pharmaceutical Incubator, University of Tehran, Iran

Research Assistant
National Institute of Genetic Engineering and Biotechnology
March 2005- February 2009
Tehran, Iran

Selected Oral Presentations

**Saatian, B.**, Austin, R. S., Kohalmi, S., Cui, Y. GLUCAN SYNTHASE-LIKE 8 regulates intercellular communication during early seedling development in Arabidopsis. CSPB/ SCBV Eastern Regional Meeting, University of Toronto, Canada, November 2015 (Ph.D. work).

**Saatian, B.**, Austin, R. S., Kohalmi, S., Cui, Y. GLUCAN SYNTHASE-LIKE 8 is required for early seedling development in Arabidopsis. CSPB/ SCBV Eastern Regional Meeting, University of Guelph, Canada, November 2014 (Ph.D. work).

**Saatian, B.**, Tian, G., Austin, R. S., Tsang, E. W. T., Kohalmi, S., Cui, Y. Next-generation mapping of seed storage protein gene repressors in Arabidopsis. CSPB/ SCBV Eastern Regional Meeting 2013, University of Toronto, Canada, December 2013 (Ph.D. work).

**Saatian, B.**, Tian, G., Austin, R. S., Tsang, E. W. T., Kohalmi, S., Cui, Y. Next-generation mapping of Arabidopsis genes. Beyond the Genome Meeting, United States, October 2013 (Ph.D. work).

Selected Poster Presentations

**Saatian, B.** Austin, R. S., Kohalmi, S., Cui, Y. GLUCAN SYNTHASE-LIKE 8 is required for early seedling development in Arabidopsis. 26th International Conference on Arabidopsis Research, France, July 2015 (Ph.D. work).

**Saatian, B.** Tian, G., Austin, R. S., Tsang, E. W. T., Kohalmi, S., Cui, Y. Create the scene and watch the show unfold: Identification of seed genes repressors in Arabidopsis Seedlings. 25th International Conference on Arabidopsis Research, Canada, July 2014 (Ph.D. work).

**Saatian, B.** Tian, G., Austin, R. S., Tsang, E. W. T., Kohalmi, S., Cui, Y. Identification of seed storage protein genes repressors in Arabidopsis seedlings using next-generation mapping. International Plant and Animal Genome XXII, United States, January 2014 (Ph.D. work).


**Saatian, B.** Mousavi, A. Detection of genetically modified seeds using PCR-based methods. 10th National Genetic Conference, Iran, May 2008 (M.Sc. work).

**Saatian, B.** Alizadeh, H., Naghavi, M. R., Omidi, M. Direct shoot regeneration of wheat (*Triticum aestivum* L.) using embryo explants and different plant growth regulators. ABIC 2004, Germany, September 2004 (M.Sc. work).

**Saatian, B.** Alizadeh, H., Naghavi, M. R., Omidi, M. Effect of plant growth regulators on direct shoot regeneration of wheat (*Triticum aestivum* L.) explants. 4th International Crop Science Conference, Australia, September 2004 (M.Sc. work).


**Saatian, B.** Alizadeh, H., Naghavi, M. R., Omidi, M. Optimization of direct shoot regeneration in wheat (*Triticum aestivum* L.) explants using different plant growth regulators. 8th Iranian Crop Production and Breeding Conference, Iran, August 2004 (M.Sc. work).