UNIVERSITY OF CALIFORNIA SAN DIEGO

Characterization of *SGT1* gene in *Physcomitrella patens*

in

Biology

by

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The dissertation of Goh Choe is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter 1, The Introduction, consists of a review of the field of auxin biology with an emphasis on biosynthesis, conjugation, transport and signaling.

Chapter 2 consists of work I have pursued during my thesis research. Mark Estelle directed and supervised the research described in this chapter.

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ABSTRACT OF THE DISSERTATION

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Professor Mark Estelle, Chair

The plant hormone auxin plays an important role in plant growth and development. Studies in flowering plants have uncovered many aspects of auxin signaling underlying molecular mechanisms. The SGT1 protein is known to be required for auxin receptor TIR1-mediated auxin response, and known to function as a HSP90 co-chaperone. However, the precise molecular function of SGT1 in auxin responses remains unknown. In this thesis, I seek to understand the function of *SGT1* in auxin responses in the moss *Physcomitrella patens* by taking advantage of its efficient recombination system. My experiments indicate that *PpSGT1* has an important role in moss development. *PpSGT1* inhibited auxin-responsive *Aux/IAA* gene expressions

when it was induced by high temperature or ectopically expressed under a strong promoter. In addition, auxin-responsive reporter expression was suppressed by *PpSGT1* overexpression. Strong growth inhibition resulting from overexpression of auxin receptor *AFB2* was compromised by *PpSGT1* overexpression. A reduction in auxin receptor accumulation in lines overexpressing *PpSGT1* implies that the reduced auxin-responsive gene expression is caused by decreased auxin receptor level. Contrarily, HSP90 appears to have a positive effect on auxin receptor accumulation and auxin reporter expression, which may suggest independent function of SGT1 and HSP90 on the auxin receptors in moss. Overall, overexpression of *PpSGT1* destabilizes auxin receptor AFB, and negatively regulates auxin signaling.

CHAPTER 1

Introduction

AUXIN

Auxin-mediated signaling controls many different biological processes during plant growth and development. Thus, tight regulation of auxin action is important for coordinating these biological processes during the plant life cycle. Roughly, the regulation of auxin response can be divided into two areas: (1) regulation of auxin concentration, and (2) regulation of auxin responsive gene expression. Plants regulate cellular auxin levels by controlling auxin metabolism, conjugation and transport. The amount of active auxin is controlled in plants by regulating auxin biosynthesis, conjugation, and degradation. Plants use a specialized transport system for auxin. Auxin influx and efflux carriers facilitate the cell-to-cell movement of auxin, to tissues where auxin function is required. In the case of auxin regulated gene expression, two protein families, the ARF transcription factors and the Aux/IAA transcriptional repressors play key roles. Auxin controls ARF activity by regulating the degradation of the Aux/IAA repressors through the ubiquitin-mediated proteolysis system.

IAA METABOLISM

It has been proposed that there are two pathways for auxin biosynthesis in plants: the tryptophan (TRP) dependent pathway and the TRP-independent pathway (Wright et al. 1991; Zhao 2010). To date, most of our understanding of auxin biosynthesis is through studies of TRP-dependent pathways, while very little is known about TRP-independent pathway. There

are four branching pathways from TRP to synthesize IAA: the tryptamine (TAM)/YUCCA (YUC) pathway, the indole-3-pyruvic acid (IPA) pathway, the indole-3-acetamide (IAM) pathway, and the indole-3-acetaldoxime (IAOx) pathway (Zhao 2010). Although the details of each TRP-dependent pathway have not been determined, recent new findings with *YUC* and *TAA1/TIR2* families have solved one of these pathways (Stepanova et al. 2008; Tao et al. 2008; Yamada et al. 2009). In this pathway, the TAA enzymes function in the first step to produce IPA from TRP. The following steps had not been defined, and the proposed model had two steps to synthesize IAA with indole-3-acetaldehyde (IAAld) as the intermediate. Recently, it was shown that the conversion of IPA to IAA can be achieved in one step, and this step is catalyzed by YUC enzymes. Thus, now we know that plants can synthesis IAA via a relatively simple processes requiring only two enzymes.

When IAA is synthesized, its activity can also be controlled by forming inactive conjugates with amino acids, peptides, or sugar. Amino acid conjugation is relatively well investigated, and some members of the GH3 family of proteins are responsible for the conjugation reaction. As storage forms of auxin, some of the IAA-amino acid conjugates (e.g. IAA-Ala, IAA-Phe, and IAA-Leu) can be hydrolyzed back to free IAA by the function of amido hydrolases (Woodward and Bartel 2005). However, IAA-Asp and IAA-Glu are considered to be irreversible IAA-amino acid conjugates, and IAA-Asp can be oxidized further for degradation (Barratt et al. 1999). Unlike Arabidopsis, IAA-

Asp hydrolysis was reported in *Medicago* (Campanella et al. 2008). Thus, there seem to be plant species-specific metabolic pathways for auxin.

AUXIN TRANSPORT

Through molecular genetics in Arabidopsis, three major classes of auxin transporters have been identified: the AUX1/LAX influx proteins, the PIN-FORMED (PIN) efflux carriers, and the ATP-binding cassette-containing (ABCB/MDR/PGP) transporters.

The AUX1/LAX proteins are auxin influx carriers. The fact that the agravitropic root phenotype of the *aux1* mutants is rescued only by membrane permeable synthetic auxins supports the influx function of AUX1 in the root (Marchant et al. 1999). Biochemical evidence for the influx activity was demonstrated in a heterologous expression system (Yang et al. 2006). In addition to gravity sensing, phylotaxis and lateral root development are controlled by this protein family (Bainbridge et al. 2008; Bainbridge et al. 2008).

The PIN auxin efflux facilitators are major determinants of the auxin distribution pattern in plants. There are eight *PIN* genes in the Arabidopsis genome. Of the eight family members, PIN1, PIN2, PIN3, PIN4, and PIN7 are localized to the plasma membrane and regulate plant development including embryogenesis, organogenesis, root meristem organization, vascular tissue differentiation, and tropic responses (Paponov et al. 2005; Tanaka et al. 2006; Zažímalová et al. 2007). The more recently characterized PIN5, PIN6, and

PIN8 are likely to localize to the endoplasmic reticulum (ER), regulating auxin levels within the cell rather than cell-to-cell auxin transport (Mravec et al. 2009). The ABCB transporters belong to the family of ATP-binding cassette transporters, which are known to be involved in trafficking processes in various organisms (Higgins 1992). Of the 29 B-type ABC transporters in Arabidopsis, 5 have been found in auxin transport inhibitor 1-naphthylphthalamic acid (NPA) binding microsomal fractions, and ABCB1, ABCB4, and ABCB19 were shown to be involved in auxin transport at the cellular level (Murphy et al. 2002; Terasaka et al. 2005; Geisler et al. 2005; Cho, Lee, and Cho 2007; Bouchard et al. 2006). Interestingly, ABCB4 expressed in HeLa cells induced IAA influx, but efflux activity in tobacco cells, suggesting that other components in plants have regulatory function of ABCB4 topology (Terasaka et al. 2005; Cho, Lee, and Cho 2007). In support of this notion, coexpression of ABCB4 and PIN1 resulted in auxin efflux, while coexpression with ABCB4 and PIN2 induced auxin influx in HeLa cell (Blakeslee et al. 2007). In Arabidopsis, *CaMV35S* promoter driven *ABCB1* expressing *AtPGP1* transgenic plants had long hypocotyls under dim white light or red light conditions (Sidler et al. 1998). The hypocotyls of *abcb1* and *abcb19* single mutants were shorter than wild type in different light conditions and had an additive interaction in hypocotyl elongation based on even shorter hypocotyls in *abcb1*;*abcb19* double mutant (Lin and Wang 2005; Nagashima et al. 2008). Consistently, auxin transport in the hypocotyl was reduced in the double mutant (Noh, Murphy, and Spalding 2001). Additionally, *abcb19* mutant has

defects in tropic response of hypocotyl in the light condition (Nagashima et al. 2008). Overall, these results suggest that ABCB1 and ABCB19 are involved in auxin-induced hypocotyl regulation in the light.

The precise distribution of auxin is required for proper organ development and differentiation, and auxin biosynthesis and polar auxin transport (PAT) contribute to the auxin distribution (Tanaka et al. 2006; Friml 2003). Because the majority of auxin is synthesized in cotyledon tips and shoot apices, auxin levels in other tissues are mainly regulated by auxin transport. In general, the localization of transporters seems to directly reflect the direction of auxin flow. Therefore, controlling polarity of auxin transporters is important for regulating auxin levels within a cell. In the root tip, different polar localization of different auxin transporters and auxin flow by them are well characterized, and some members also show different localizations in different tissues. For example, in the root tip, AUX1 is localized symmetrically in the columella and lateral root cap, but asymmetrically in the protophloem (Swarup et al. 2001). PIN1 localizes at the basal side of vascular tissue and stele in the root, whereas PIN2 localizes at the apical side of epidermal and basal side of cortex cells (Blilou et al. 2005). PIN3 shows uniform distribution in columella cells, but localizes at the lateral, inner side of the pericycle (Friml et al. 2002). Together with PIN4 and PIN7, the localization of these PIN proteins suggests that an auxin reflux loop acts in the root, and revealed the relationship between auxin flux and root pattern formation (Blilou et al. 2005).

In the hypocotyl, PIN1 immunolocalization signal was seen at the basal end of the vascular tissue, cortical, and bundle sheath cell files (Noh et al. 2003). However, GFP-fused PIN1 showed expression only in the vascular tissue in light grown seedlings and epidermal and cortical cells in dark seedlings, requiring more experiments to clarify this conflict. PIN7 showed apolar localization in the epidermal cells of the hypocotyl (Blakeslee et al. 2007). PIN3 localized to the lateral side of bundle sheath cells consistent with its role in tropic responses (Friml et al. 2002). In the case of ABCB19, the localization in the vascular tissue, bundle sheath, and cortical cells partly overlaps either with PIN1 or PIN3 (Blakeslee et al. 2007). It has been reported that ABCB19 and PIN1 have a synergistic effect on auxin transport and increase substrate specificity when they were expressed in the same HeLa cells (Blakeslee et al. 2007; Noh et al. 2003). Similarly, an interaction between ABCB1 and PIN2 was suggested (Blakeslee et al. 2007). However, the different subcellular localization of PIN and ABCB proteins in the hypocotyl implies the possibility of different combination of PIN and ABCB protein interactions, but experimental evidence is lacking. Localization of auxin transporters is dynamically regulated by developmental and environmental cues. For example, PIN3 in the columella cells relocalizes to the bottom side when there is a new gravity force (Friml et al. 2002). In the case of PIN7, localization at the apical side of suspensor cells mediates auxin flow into the young embryo. However, at later stages of embryo development, PIN7 relocalizes at the basal side of the suspensor cells, redirecting auxin flow in

the opposite direction thus contributing to root tip specification (Friml 2003). ABCB proteins have been shown to localize in an apolar manner in the root tip, but ABCB1 and ABCB4 localize polar in the mature root in certain cases (Cho, Lee, and Cho 2007; Blakeslee et al. 2007; Geisler et al. 2005).

Phosphorylation is known to contribute to polar targeting of PIN proteins. Serine/Threonine kinase PINOID (PID) overexpressing plants induces a shift in PIN localization from the basal to apical membrane, whereas *pid* leads to the opposite localization (Friml et al. 2004). Protein phosphatase 2A (PP2A) has antagonistic effects on PID (Michniewicz et al. 2007). Cellular trafficking is another regulatory mechanism of auxin transport proteins. According to a recent report, PINs are initially localized at the plasma membrane without polarity and membrane trafficking processes establish their polar localization (Dhonukshe et al. 2008). In the earlier reports, GNOM, an endosomal exchange factor for ARF GTPases (ARF GEF) is involved in the vesicle trafficking processes of PIN1 (N. Geldner et al. 2001). GNOM targeting Brefeldin A (BFA) interferes with PIN1 excocytosis, forming BFA compartment. In contrast BFA insensitive GNOM suppresses BFA induced PIN1 internalization (Niko Geldner et al. 2003). PIN2 is targeted by the SORTING NEXIN1-dedicated trafficking pathway(Jaillais et al. 2006). In the hypocotyl, PIN1 localization is likely to be regulated in a light-dependent manner. Basal localization of PIN1 in the hypocotyl on the distal side to the light source seems to be destabilized by blue light treatment and the light effect is missed

in the blue light insensitive *phot1* mutant (Blakeslee et al. 2004). However, little is known about the transporter targeting in the hypocotyl.

AUXIN SIGNALING

The transduction of the auxin chemical signal to gene expression occurs in the nucleus by the interaction of three protein families: the TIR1/AFB F-box proteins, the ARF transcription factor family, and the Aux/IAA transcription repressor family. The signaling pathway for early auxin responsive genes is relatively simple. The ARF transcription factors can target auxin responsive gene through short DNA element called auxin response element (AuxRE) (Ulmasov, Hagen, and Guilfoyle 1997). The canonical AuxRE sequence, TGTCTC, was identified from the promoter analyses of soybean GH3 and pea IAA4/5 genes and also found in the promoter of many auxin responsive genes (Ballas et al. 1995; Ballas, Wong, and Theologis 1993). The activity of ARFs is suppressed by the Aux/IAA repressors. Interaction between ARF and Aux/IAA proteins occurs in their conserved C-terminal domains called domain III and IV (Gretchen Hagen and Guilfoyle 2002). The Aux/IAA proteins have two additional conserved domains called domain I and II (Gretchen Hagen and Guilfoyle 2002). In domain I, there is at least one conserved motif called the EAR motif (ethylene response factor-associated amphiphilic repression) which recruits the TOPLESS transcriptional corepressor to prevent ARF mediated transcriptional activation (Szemenyei, Hannon, and Long 2008). Auxin responsiveness can be achieved by

conditional derepression of ARFs by auxin and the TIR1/AFB F-box proteins (Gray et al. 2001). When auxin is present, auxin binds to the TIR1/AFB protein to create an interaction domain for the degron motif in domain II of the Aux/IAA proteins (Dharmasiri, Dharmasiri, and Estelle 2005; Kepinski and Leyser 2005). This leads to the ubiquitination of the Aux/IAAs and their degradation by the 26S proteasome. With this, ARF transcription factors are released from the repression by Aux/IAAs. Many genes are activated through this process including some members in Aux/IAA, ACS, GH3, and SAUR gene families, which were among the first characterized auxin responsive genes (Abel and Theologis 1996). This process doesn't require *de novo* protein synthesis, thus some auxin responsive genes can be induced within 5 minute of auxin treatment (G Hagen and Guilfoyle 1985). Microarray experiments have identified many more genes that respond to auxin. They are involved in different cellular processes including hormone regulation, signal transduction, and cell expansion (Sawa et al. 2002; Pufky et al. 2003; Zhao et al. 2003; Armstrong et al. 2004; Himanen et al. 2004; Nemhauser, Mockler, and Chory 2004; Redman et al. 2004; Nagpal et al. 2005; Okushima et al. 2005; Overvoorde et al. 2005; Vanneste et al. 2005; Nemhauser, Hong, and Chory 2006; Paponov et al. 2008; Chapman et al. 2012). In addition, many of them showed overlapping transcriptional responses of different signals such as hormones, pathogen attack, or light conditions *etc*. Thus, further studies need to be carried out to characterize their roles in auxin signaling.

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CHAPTER 2

Characterization of *SGT1* gene in *Physcomitrella patens*

ABSTRACT

The HSP90 co-chaperone SGT1 is known to be involved in auxin signaling. In moss *Physcomitrella patens*, the *SGT1* homolog *PpSGT1* is a single copy gene, and a *ppsgt1* knockout mutant appears to be lethal. As an alternative, the effects of *PpSGT1* overexpression were investigated. In the *PpSGT1* overexpression lines, colony growth was slightly inhibited. Auxin-responsive *IAA1a* gene expression, and auxin-responsive *DR5:DsR* reporter expression was also reduced. *PpSGT1* expression was induced by high temperature conditions for 2 or 24 hours, and *IAA1a* and *IAA1b* gene expression were negatively regulated after a 24-hour high temperature treatment. The effects of *PpSGT1* on auxin receptor *AFB2* were analyzed with transgenic mosse lines overexpressing both *PpSGT1* and *AFB2*. In the overexpression lines, colonies showed hyposensitive responses to auxin, and AFB2 protein levels were decreased. The role of HSP90 on auxin responses was analyzed using the HSP90 specific inhibitor geldanamycin (GDA). GDA inhibited auxin-induced *DR5:DsR* reporter expression, and AFB2 accumulation. Collectively, *PpSGT1* negatively regulates auxin responses when it is overexpressed, while HSP90 has a positive role.

INTRODUCTION

The ubiquitin-mediated proteolysis system is a core component of auxin signal transduction. The SCF^{TIR1} E3 ligase functions as an auxin receptor and

promotes the degradation of the Aux/IAA repressors. SCF^{TIR1} also contains three additional core subunits. SKP1 is an adapter linking the F-box protein to the Cullin subunit. Cullin also binds the RBX protein (Ring-Box protein) that recruits ubiquitin-loaded E2 enzyme to the SCF complex. Although these three SCF subunits have relatively smaller numbers of family members and are likely to be shared by large number of F-box proteins, the loss of these subunits can still affect auxin signaling (Gagne et al. 2002). Indeed, Arabidopsis SCF subunit mutants such as *ask1-1* (*Arabidopsis SKP1-like1*), *Cullin1* allele *axr6-1* (*auxin resistance 6-1*) or *axr6-2* showed reduced auxin responsiveness in root elongation or hypocotyl growth, and had various phenotypic defects related to auxin including reduced number of lateral roots, incomplete vascular patterns, or shorter inflorescences (Gray et al. 1999; Hobbie et al. 2000; Hellmann et al. 2003) .

To form a functionally active SCF ubiquitin ligase complex, further modification of Cullin subunit is required. NEDD8 is a small ubiquitin-like protein. It is attached to the Cullin subunit through a process called neddylation that is similar to ubiquitination. Nedd8 conjugation induces conformational changes of Cullin that facilitates ubiquitin transfer from the E2 to the substrate proteins. The conjugated NEDD8 is removed from Cullin by the COP9 signalosome complex (CSN) which was originally identified from mutant screens for constitutive photomorphogenic responses (Wei, Chamovitz, and Deng 1994). It contains eight subunits, and is homologous to

the 19S lid complex of the 26S proteasome complex (Wei, Chamovitz, and Deng 1994). The deneddylation activity of CSN resides in CSN5 subunit (Cope et al. 2002). There is another regulatory protein that is associated with the neddylation and deneddylation cycle. CAND1 (Cullin-associated-Nedd8-dissociated-1) regulates SCF function by interacting with the Cullin subunit (Bosu and Kipreos 2008). CAND1 binds to Cullin that is not modified by NEDD8, and blocks SKP1 and NEDD8 binding sites. In turn, NEDD8 conjugation interferes with CAND1 interaction with Cullin (Liu et al. 2002; Zheng et al. 2002). The function of CAND1 had long remained a puzzle since it binds to unneddylated Cullin, yet based on genetic data, promotes SCF activity (Liu et al. 2002; Zheng et al. 2002; Goldenberg et al. 2004; Feng et al. 2004; Kim et al. 2010). Recently, it has been suggested that CAND1 acts as a substrate receptor exchange factor to timely process different SCF substrate in response to cellular signal changes (Pierce et al. 2013). As a regulatory component of SCF complexes, various developmental defects were observed in *cand1* mutants including delayed flowering time, floral organ defects, and constitutive photomorphogenesis, *etc* (Chuang, Zhang, and Gray 2004; Feng et al. 2004). *CAND1* (*ETA2*) was also identified in an enhancer screen of the *tir1-1* mutant (Chuang, Zhang, and Gray 2004). Root growth inhibition by auxin was weakly compromised in the single *cand1* mutants, but the double mutant with *tir1-1* exhibited strong auxin resistance (Chuang, Zhang, and Gray 2004). In addition, auxin responsive reporter expression was poorly induced, and

IAA7/AXR2 protein accumulated more in *eta2-1* mutant (Chuang, Zhang, and Gray 2004).

The *Arabidopsis sgt1b (eta3)* mutation is another genetic enhancer of *tir1-1* (Gray et al. 2003). Auxin-mediated responses including 2,4-D resistance on primary root growth, lateral root initiation, and high temperature-mediated hypocotyl growth were enhanced in the *eta3 tir1-1* double mutant compared with *tir1-1* single mutant (Gray et al. 2003). SGT1 was first identified as a suppressor of the *skp1* mutant in *Saccharomyces cerevisiae* (Kitagawa et al. 1999). SKP1 together with NDC10, CEP3, and CTF13 forms a CBF3 kinetochore subcomplex, and SGT1 and SKP1 are known to activate F-box protein CTF13 for CBF3 assembly (Kitagawa et al. 1999). SKP1 is not only associated with SGT1 interaction. SGT1 directly interact with adenylyl cyclase activation in yeast (Dubacq et al. 2002). In plants, SGT1 is known to interact with RAR1 (Required for Mla12 resistance 1), and contributes to resistance (R) protein-mediated pathogen response (Azevedo et al. 2002; Austin et al. 2002). Later, SGT1 and R protein interaction was also observed by yeast two-hybrid assay (Bieri et al. 2004). Mammals also have R-protein related NB-LRR (nucleotide binding-leucine rich repeat) domain containing proteins (also called NLR), and interaction between SGT1 and NLR proteins has been observed (Mayor et al. 2007). The *Salmonella* novel E3 ubiquitin ligase (NEL) SspH2 is a newly added SGT1 interacting protein (Bhavsar et al. 2013). SspH2 has

been shown to activate immune responses by monoubiqutinating NLR protein NOD1 (Bhavsar et al. 2013).

The common link of many of these SGT1 interacting partners is HSP90 (heat shock protein 90). HSP90 is required for SGT1 and SKP1 interaction, and promotes SKP1 interaction with CTF13 (Bansal, Abdulle, and Kitagawa 2004; Stemmann et al. 2002; Bansal et al. 2009). Physical interaction between SGT1, HSP90, and adenylate cyclase was observed in the fungal pathogen *Candida* (Shapiro et al. 2012). HSP90 together with SGT1 is required for R or NLR-mediated immune responses in plants and mammals (Hubert et al. 2003; Mayor et al. 2007). HSP90 is a molecular chaperone which is required for the maturation and activation of client proteins which function in many different cellular processes including signal transduction, intracellular transport, and protein degradation (Shirasu 2009). The activity of HSP90 is assisted by different types of co-chaperones. They have many different roles such as recruiting their specific client proteins to the HSP90, regulating the ATPase activity of HSP90, translocating client proteins into different cellular compartment, and targeting proteins for degradation (Li, Soroka, and Buchner 2012).

SGT1 is one of the co-chaperone having a domain with structural similarity to the well-known HSP90 co-chaperone p23, but they do not share a common binding site and function (Shirasu 2009). SGT1 is highly conserved in eukaryotes. SGT1 contains five domains including the TPR (tetratricopeptide

repeat) domain, the CS (present in CHP and SGT1 protein) domain, the SGS (SGT1-specific) domain, and two variable regions (VR1 and VR2) (Azevedo et al. 2002). However, The TPR domain is missing in the SGT1 of *Drosophila*, *Caenorhabditis*, and *Brugia* (Shirasu 2009; Martins et al. 2009). The TPR domain has known to be required for SKP1 interaction as a dimer for kinetochore assembly in yeast (Bansal, Abdulle, and Kitagawa 2004; Bansal et al. 2009). However, in plants, the TPR domain is dispensible for plant immune responses and auxin signaling (Azevedo et al. 2006). Interestingly, many HSP90 co-chaperones share TRP domain, and bind to the C-terminal MEEVD tail of HSP90 through the TPR domain for their function (Li, Soroka, and Buchner 2012). In the case of SGT1, the TPR domain was thought to contribute to HSP90 interaction initially, but later it was shown that the CS domain is sufficient for the interaction with HSP90 (Catlett and Kaplan 2006; Lee et al. 2004). The CS domain also interacts with RAR1 CHORD-II domain to form a ternary complex with HSP90 (Azevedo et al. 2002; Botër et al. 2007; Zhang et al. 2010).The C-terminal SGS domain is the most highly conserved region of SGT1 (Azevedo et al. 2002). It is required for the interaction with the LRR domain of adenylate cyclase, NLR proteins, and SspH2 which seem to be HSP90 clients (Bhavsar et al. 2013; Bieri et al. 2004; Dubacq et al. 2002). HSP70 is also known to interact with the SGS domain of SGT1 *in vivo* (Noël et al. 2007). The binding nature of HSP70 and SGT1 is not known yet, but their association could be indirect because no *in vitro* or yeast two-hybrid interaction has been observed (Noël et al. 2007). Some members of the S100

family, which contain calcium binding EF-hand motifs, has been shown to interact with SGS domain of SGT1 (Donato 1999; Nowotny et al. 2003). These proteins are also thought to regulate the phosphorylation of their substrates by blocking the phosphorylation sites (Donato 2001). Recently, it was reported that SGT1 phosphorylation status is important for its subcellular localization. Arabidopsis SGT1 with a mutation for threonine at position 346 with aspartate to mimic phosphorylated form preferentially localized in the nucleus (Hoser et al. 2013). *eta3* mutant which expresses a truncated SGT1 missing the last 36 amino acid including the Thr346 localized only in the nucleus-depleted fraction, suggesting that the Thr346 phosphorylation is required for SGT1 nuclear localiazation (Noël et al. 2007). In human, however, non-phosphorylatable SGT1 mutant localized in the nucleus, and phosphomimic SGT1 was depleted from the nuclear compartment (Prus et al. 2011). Thus, further analyses are required to fully understand the role of S100 proteins on SGT1.

SGT1 is known to be required for the accumulation of many NLR proteins, such as tobacco Rx protein and N protein (Botër et al. 2007; Leister et al. 2005; Mestre and Baulcombe 2006). However, SGT1 is likely to have the opposite effect on *Arabidopsis* RPS5 (Holt, Belkhadir, and Dangl 2005). In other cases, SGT1 only affects the activation of HSP90 clients with no influence on their stability, while HSP90 is required for the protein stability (Correia et al. 2007; Shapiro et al. 2012). Thus, further studies are needed to understand the relationship between SGT1 and HSP90 client proteins.

In *Arabidopsis*, there are two *SGT1* genes, *SGT1a* and *SGT1b*. Of these, only *SGT1b* was isolated in several mutant screens for pathogen responses or auxin signaling (Austin et al. 2002; Gray et al. 2003; Muskett et al. 2002). SGT1a seems to be unstable, and the two threonine residues at position 91 and 100 in the TPR domain which are alanines in other SGT1 proteins in plants are responsible for the instability (Azevedo et al. 2006). When SGT1a level increased by strong promoters or TPR domain of SGT1a was modified by the domain swap with SGT1b or the site-directed mutagenesis of both Thr91 and Thr100 to alanines, SGT1a could complement the defects in *sgt1b* mutant for auxin and pathogen responses, suggesting functional redundancy between the two paralogs (Azevedo et al. 2006). The embryo lethal phenotype of the *sgt1a sgt1b* double mutant also supports that (Azevedo et al. 2006).

The moss *Physcomitrella patens* is a simple non-vascular land plant which diverged from the flowering plant lineage approximately 450 million years ago (Lang et al. 2008). Compared with higher plants, it has haploid-dominant life cycles with two different developmental gametophyte stages: the filamentous tissue called protomema and the leafy tissue called gametophore. The protonemal tissue first produced from the haploid spore is called chloronema which is characterized by a large number of chloroplasts and cross walls at right angles to the filament axis. Later, chloronemal cells at the tip of the filaments differentiates into the second type of protonemal cell called caulonema. These cells contain fewer chloroplasts and oblique cross walls

between cells in the filaments. Side branch initial cells are formed from the subapical cells of the primary protonemal tissues to produce secondary protonemal filaments. Some branch initials from caulonemal cells differentiate into buds that give rise to gametophores with leafy shoots. At the base of the gametophores, brown-pigmented root-like protonemal filaments called rhizoids are produced, and support the aerial part of the gametophores (Figure 2.1).

Despite the differences in morphology and life cycle between the moss and flowering plants, many genes involved in signaling processes in flowering plants are present in the moss genome, which makes the moss a useful model system for the functional studies of genes and their evolution in land plants (Rensing et al. 2008). It also has other features suitable as a model organism: small size, short life cycle, simple vegetative propagation for cultivation, and sequenced genome information (Cove et al. 2009). Most importantly, efficient gene targeting by homologous recombination is a unique advantage compared with the *Arabidopsis* system (Schaefer and Zrÿd 1997).

The *Physcomitrella* genome encodes gene families involved in primary auxin responses in higher plants including three Aux/IAA, fifteen ARF, and four TIR1/AFB proteins (Rensing et al. 2008; Paponov et al. 2009; Prigge et al. 2010). Homologs of auxin biosynthesis and conjugation genes are present as well. However, there are some differences between mosses and flowering plants. Homologous genes that are responsible for auxin conjugate hydrolysis are not present in the moss genome (Ludwig-Müller 2011). Polar auxin transport was reported in rhizoids and sporophytic tissues, but not in the

gametophytic shoot (Sakakibara et al. 2003; Fujita et al. 2008). During moss development, auxin induces the transition from chloronema to caulonema in the protonema stage, and during stem elongation, the rhizoid development in the gametophore stage (Ashton, Grimsley, and Cove 1979; Fujita et al. 2008). Understanding the underlying molecular mechanisms that regulate these developmental changes in moss is still in the early stages. However, homologs of *Arabidopsis* genes known to be involved in the auxin signaling seem to be responsible for some of the developmental processes. *ROOT HAIR DEFECTIVE SIX-LIKE* (*RSL*) genes are bHLH transcription factors induced by auxin, and control root hair development in Arabidopsis (Masucci and Schiefelbein 1994). Recently, it was reported that auxin-induced caulonema differentiation is controlled by the *PpRSL1* and *PpRSL2* genes, moss homologs of *Arabidopsis RSL* genes (Menand et al. 2007). In the *Pprsl1* and *Pprsl2* double mutant, only chloronemal filaments were produced, and caulonema differentiation was not induced even in the presence of auxin (Menand et al. 2007; Jang and Dolan 2011). Mutants with defects in *Aux/IAA* genes and *AFB* auxin receptor knock-down lines showed developmental arrest at the chloronemal stage as well (Ashton, Grimsley, and Cove 1979; Prigge et al. 2010). *PpSHI* genes, homologs of the Arabidopsis *SHI/STY* genes which are known to activate YUC4 expression, induced caulonemal tissue production and gametophore development earlier than the wild type mosses when they were overexpressed (Eklund, Ståldal, et al. 2010; Eklund, Thelander, et al. 2010). *DIAGEOTROPICA* (*DGT*) encodes a type A cyclophilin which is known

to affects the expression of auxin-responsive genes (*Aux/IAAs* and *SAURs*) and auxin-associated developmental responses (lateral root development, gravitropic responses, apical dominance, and fruit development) in tomato (Rice and Lomax 2000; Balbi and Lomax 2003; Ivanchenko et al. 2006). Moss *PpDGT* also contribute to auxin-responsive gene expression and *ppdgt* knockout mutants exhibited delayed or insensitive responses to auxin in the *ppdgt* mutants (Lavy et al. 2012).

RESULTS AND DISCUSSION

Phylogenetic analysis of SGT1 gene in plants

The relationship of the moss *SGT1* (*PpSGT1*) to other plant *SGT1* genes in 23 plant species was analyzed with budding yeast *SGT1* as an outgroup (Figure 2.2). The complete coding sequence of each gene with all the three domains (TPR, CS, and SGS domain) was included in the phylogenetic analysis. *SGT1* exists as a single copy in moss, and diverged early from other land plant *SGT1* homologs. *SGT1* genes in the flowering plants shown are separated into two clades which contain sequences from monocots and dicots respectively, and two or more paralogs of *SGT1* exist in some species. The dicot clade forms two major clusters, 1) a cluster containing crucifer species, legume species, and one caster bean *SGT1*, 2) a cluster containing the other asterids and rosids species used in the tree analysis and the other caster bean *SGT1*. It appears that there exist at least two different *SGT1* proteins early in the flowering plant evolution, and each

cluster lost a different member of *SGT1* during their evolution, while caster bean maintained both *SGT1* proteins. Thus, *SGT1* paralogs in each species except the caster bean appear to be originated from a relatively recent genome or gene duplication events. In the crucifer plants, one duplication event seemed to happen before their speciation, then the *Brassica* had its own *SGT1* duplication afterwards.

In this phylogenetic analysis, *SGT1* homologs with all three conserved domains were analyzed. Thus, it is possible that there are more SGT1-like proteins that can produce functional proteins but have only partial homology to the full length SGT1 as in some anthropods and nematodes. The TPR domain has shown to be dispensable for immune responses and auxin signaling in *Arabidopsis* as well. CacyBP (Calcyclin binding protein/Siah-1-interacting protein) is a homolog of SGT1 containing the CS and the SGS domains, two functionally important domains in *SGT1*. It interacts with SKP1 and S100 family proteins which are also known to interact with SGT1 (Filipek and Kuźnicki 1998; Matsuzawa and Reed 2001). In addition, CacyBP has been shown to function as a component of a ubiquitin ligase complex which regulates phosphorylation-independent beta-catenin degradation in human (Matsuzawa and Reed 2001). Although SGT1 and CacyBP share many common features. *sgt1a sgt1b* double mutant showed embryo lethality in *Arabidopsis*, suggesting that they have diverged function in plant development. Thus, gene structures homologous to *SGT1* in the genome may not have a conserved function.

PpSGT1 knock-out mutant

To understand the function of *PpSGT1* in moss, the targeted knock-out of *PpSGT1* was attempted. Two different constructs were used to isolate *ppsgt1* knock-out moss lines (Figure 2.3). The construct for *ppsgt1-1* replaces the third, fourth, and fifth exons with an antibiotic resistance cassette (Figure 2.3A). In the case of *ppsgt1-2*, the targeting vector will replace the whole gene body with a hygromycin resistance gene cassette leaving no structural domains of PpSGT1 (Figure 2.3B). These two constructs were inserted into the wild-type moss genome through PEG-mediated protoplast transformation technique. After two rounds of antibiotic selection and non-selection cycles, about 100 colonies with hygromycin resistance were obtained. The colony phenotypes of these colonies can be categorized into three groups roughly. The first group had small and chloronema-rich colonies. The second group were not distinguishable from wild-type colonies. The third group had longer protonema filaments with few branches. However, genotyping PCR to check the genomic DNA structure of these putative mutant lines showed no discernable patterns between them. In all the hygromycin resistant mosses isolated, mutants with homologous recombination on both ends were not obtained. Many of them had a non-homologous end-joining event at one of the homologous target sites. In moss, there is a chance that the knock-out mutant of *PpSGT1* is lethal because there is only one gene in the moss genome. In

Arabidopsis, there are two alleles of *SGT1*, and the double mutant *sgt1asgt1b* is embryo lethal (Azevedo et al. 2006).

SGT1 overexpression phenotype

To gain insight into the role of SGT1 on auxin signaling, transgenic moss lines overexpressing *PpSGT1* were generated. This line expresses *PpSGT1* under the control of the maize ubiquitin promoter at the *Pp108* locus in moss (Schaefer and Zrÿd 1997; Vidali et al. 2009). Fifteen stably transformed lines were isolated and examined for their *SGT1* expression levels. qRT-PCR results show that *SGT1* transcript levels were significantly increased in the transgenic lines (Figure 2.4A). Although the expression of *SGT1* is regulated by the same promoter, moss lines with different *SGT1* transcript levels were obtained, suggesting that non-specific integration or concatenation of the transforming DNA had occurred (Kamisugi et al. 2006). Protein expression levels based on immunoblot analysis also showed varying levels of SGT1 expression in different transgenic lines, and correlated with qRT-PCR result (Figure 2.4B). Line #13, 14, and 15 with the highest levels of PpSGT1 also had additional bands with smaller sizes. These bands could be the result of partial degradation of PpSGT1 or translation of truncated *SGT1* mRNA originated by non-homologous recombination into the moss genome. Line #1, #17, and #20 with modest *SGT1* overexpression and line #13, #14, and #15 with highest *SGT1* overexpression were chosen for further characterization. Lines #1, #7, and #20 did not have significant differences in

their colony phenotype 14 days after subculture onto fresh medium. However, line #13, #14, and #15 had slightly smaller colony sizes with shorter caulonemal filaments compared with wild type (Figure 2.5A).

To examine whether the *PpSGT1* overexpressing lines have phenotypic differences in later stages of moss development, colonies were grown for 4 weeks. Similar to 14 day-old mosses, line #1, #7, and #20 had no apparent morphological differences from a wild-type moss colony. However, line #13, #14, and #15 remained small, and had a smaller number of gametophores. Thus, the number of gametophores was counted at this stage in wild-type and transgenic moss lines. Compared with wild type, *PpSGT1* overexpressing mosses developed reduced number of gametophores (Figure 2.5B). The result also showed a negative correlation between the *SGT1* expression level and the gametophore numbers with less gametophore development in higher *PpSGT1* expressing mosses. This suggests that *PpSGT1* has a negative role in gametophore development. Auxin is known to affect protonema and gametophore development. In the presence of auxin, mosses develop less protonemal tissues, advance to the caulonemal stage earlier, and then eventually form less gametophores with rhizoid filaments instead of leafy structures (Jang and Dolan 2011). Both SGT1 overexpression and auxin application inhibit gametophore development. Thus, there is a possibility that *PpSGT1* enhances auxin-mediated developmental changes in moss. However, it is also possible that *SGT1* overexpression caused developmental defects independent of auxin responses.

The effects of *PpSGT1* on the development of moss in response to auxin

To determine how over-expression of *PpSGT1* affects auxin responses in moss, wild type and *PpSGT1* overexpressing moss lines were grown on auxin containing medium. Auxin inhibits moss growth and development in a concentration-dependent manner with strong growth inhibition at 12.5 µM NAA or higher (Ashton, Grimsley, and Cove 1979). Wild-type moss grown for one month on 0.5 µM NAA showed modest growth inhibition with reduced colony size, smaller numbers of gametophores, and increased brown pigmentation. At 12.5 µM NAA, growth was further inhibited with small colony size with almost no gametophore development (Figure 2.6). If *PpSGT1* overexpression has a positive effect on auxin signaling causing delayed growth and inhibition of gametophore development, enhanced auxin sensitivity would be expected. After one month on control medium, *PpSGT1* overexpressing lines had increased brown pigmentation in the absence of auxin except *PpSGT1* overexpressing line #1. However, no significant morphological changes were observed in response to auxin, such as severe colony growth inhibition or the transition of gametophore leaflets to rhizoid-like structures in the absence or in the presence of low concentration of auxin. Thus, *PpSGT1* overexpression does not seem to affect auxin-mediated developmental changes significantly.

The effect of *PpSGT1* on auxin response gene regulation in moss

Auxin is known to regulate gene expression by derepression of ARFs through TIR1/AFB-mediated ubiquitination of AUX/IAA repressor proteins (Gray et al. 2001). *GH3, AUX/IAA*, and *SAUR* genes are well-known auxin regulated gene families (Abel and Theologis 1996). In moss, *AUX/IAA* genes are induced by auxin treatment, while *SAUR* gene expression is known to be reduced by auxin (Lavy et al. 2012). To further characterize the effect of *PpSGT1* overexpression on auxin responses in moss, auxin responsive *IAA1a* gene expression was analyzed (Figure 2.7). In the *PpSGT1* overexpressing lines, auxin induction *IAA1a* was not affected significantly with a similar level of induced expression in the presence of auxin in all transgenic lines with. Instead, the basal level of *IAA1a* expression seems to be affected by *PpSGT1* overexpression. Line #13 and #14 which exhibit a high-level of *PpSGT1* overexpression showed reduced *IAA1a* expression both in the absence and presence of auxin compared with the wild type. Line #15 also had high-level of PpSGT1 protein expression, but showed minor reduction of *IAA1a* expression. Line #1 and #20 with a lower level of *PpSGT1* expression than line #13, #14, and #15 had similar *IAA1a* expression with slightly reduced *IAA1a* induction in response to auxin. These results suggest that *PpSGT1* does not have a major role in auxin mediated signal transduction in moss. However, reduced *IAA1a* transcript levels with a high level of PpSGT1 may suggest that could affect some aspect of the auxin signaling pathway, and the AFB auxin receptors are possible candidates. If *PpSGT1* affects AFB protein accumulation or function, AFB ubiquitin E3 ligase substrate Aux/IAA levels and *Aux/IAA* transcript levels

can be affected. PpSGT1 seems to have a negative effect on AFB function based on the *IAA1a* gene expressions. However, it is possible that *PpSGT1* has positive effects on *AFBs* or other upstream components because of the feedback regulation of *Aux/IAA* genes. Aux/IAAs repress ARF activity, but their expression is induced by ARFs. Thus, analysis of auxin responsive reporter expression or quantification of AFB level is required to interpret the result more clearly.

To further confirm the effects of *PpSGT1* overexpression on auxin response gene expression, *PpSGT1* was overexpressed in the background of a transgenic moss expressing an auxin responsive reporter NLS4;*DR5:DsR*. NLS4;*DR5:DsR* expresses two fluorescence reporter proteins. GFP is constitutively expressed under control of the *35S* promoter, and DsRed2 under the auxin responsive *DR5* synthetic promoter. *PpSGT1* overexpressing expression construct was inserted into the *Pp108* locus by homologous recombination. qRT-PCR result shows that *SGT1* transcript level was significantly increased in the transgenic lines (Figure 2.8A). In the *PpSGT1* overexpressing lines and NLS4;*DR5:DsR,* auxin response was analyzed. For quantitative analysis, protoplasts were isolated from protonemal tissues after 48 hour incubation in BCD liquid medium with or without 12.5 µM NAA, and the ratio of DsRed intensity relative to GFP intensity was measured to evaluate auxin response with a flow cytometer. *PpSGT1* overexpressing line #9 and line #14 exhibited reduced auxin reporter induction in response to auxin (Figure2.8B). *PpSGT1* overexpressing line #9 has no difference from the

NLS4;*DR5:DsR* control in the absence of auxin, which may suggest that *PpSGT1* overexpression has negative role in auxin signaling in moss. However, *PpSGT1* overexpressing line #14 has significant reduction both in the absence and the presence of auxin. Thus, more transgenic lines need to be analyzed to confirm the negative regulation of *PpSGT1* on auxin signaling. Colony phenotypes of the transgenic mosses were observed to analyze their auxin response phenotypes (Figure2.8C). *PpSGT1* overexpressing line #9 had no significant differences compared with the NLS4;*DR5:DsR* control. However, the line #14 had much smaller colony growth at high concentration of auxin. Thus, more transgenic lines need to be isolated, and other auxin response markers need to be analyzed to draw a conclusion about the effect of *PpSGT1* overexpression on auxin signaling. However, both transgenic lines showed decreased auxin reporter expression, suggesting that *PpSGT1* overexpression has a negative effect on auxin signaling.

The effect of *PpSGT1* on auxin receptor levels in moss

In figure 2.7, reduced *IAA1a* level was observed in the strong *PpSGT1* overexpressing lines. To determine if this reduced *IAA1a* expression is caused by changes in *AFB* levels, the *ubi:PpSGT1* construct was introduced into a line over-expressing *AFB2-myc*. Two independent *PpSGT1* overexpressing lines were analyzed for their *PpSGT1* expression level using qRT-PCR. Both line #7 and line #10 had increased *PpSGT1* transcript levels with higher *PpSGT1* expression in line #10 (Figure 2.9A). In these *PpSGT1* overexpressing lines

and *Pubi:AFB2myc*, AFB2-myc protein level was analyzed by immunoblot assay using myc antibody. In both of the *PpSGT1* overexpressing lines, AFB2-myc level were reduced compared with *AFB2-myc* overexpressing moss *Pubi:AFB2myc* (Figure2.9B). Unexpectedly, *PpSGT1* overexpressing line #10 with higher amount of *PpSGT1* transcript than *PpSGT1* overexpressing line #7 showed less reduced accumulation of AFB2-myc protein than *PpSGT1* overexpressing line #7. Thus, this needs to be further confirmed and SGT1 protein levels need to be analyzed. However, in general, *PpSGT1* overexpression caused reduced AFB2-myc accumulation in moss.

The effect of PpSGT1 overexpression on AFB2 protein function was further characterized by analyzing colony growth phenotypes and developmental changes by auxin. Compared with wild type, *Pubi:AFB2myc* moss expresses high amount of AFB2-myc. Thus, *Pubi:AFB2myc* shows hypersensitive response to auxin by showing strong growth defects at a lower concentration of auxin which has weak growth inhibitory effects on wild type plants. In the absence of auxin, *PpSGT1* overexpressing lines in the *Pubi:AFB2myc* looked similar to *Pubi:AFB2myc*. In the presence of auxin, however, *PpSGT1* overexpressing lines showed larger colony sizes than *Pubi:AFB2myc* having more gametophore structures(Figure 2.9C). It seems that *PpSGT1* overexpressing lines in the *Pubi:AFB2myc* background have reduced auxin hypersensitivity with less AFB2-myc accumulation than *Pubi:AFB2myc*. Thus, it appears that *PpSGT1* overexpression has negative effects on AFB function and AFB mediated colony growth responses in the

presence of auxin. However, this negative effect on auxin responses is only observed in growth conditions with exogenous auxin and in the AFB2 overexpressing moss. In the absence of auxin, *PpSGT1* overexpressing lines in the *Pubi:AFB2myc* background had no conspicuous phenotypic changes. Other auxin signaling mutants in moss show strong phenotypes with developmental arrest in early protonemal stages forming round shaped chloronema-rich colonies (Lavy et al. 2012; Prigge et al. 2010). Such a severe developmental defect was not observed in *PpSGT1* overexpressing lines. In the wild type background, PpSGT1 overexpression has contrasting effects, Transgenic moss with strong *PpSGT1* overexpression had smaller colony sizes and reduced gametophores than wild type, which is morphologically more similar to mosses grown in the presence of auxin. Of the moss *aux/iaa* mutants reported, *ppiaa2-87s7* which has a premature stop codon in the upstream of domain II has similar colony phenotype with small size and a few gametophores (Prigge et al. 2010). Thus, it is possible that the small colony phenotype by *PpSGT1* overexpression in the wild type background is also caused by reduced auxin responses. Based on these results, *PpSGT1* overexpression seems to have minor inhibitory effects on auxin signaling. In *Arabidopsis*, *SGT1* seems to have positive function in auxin responses because *tir1sgt1b* double mutant showed reduced sensitivity to synthetic auxin 2,4-D in primary root elongation (Gray et al. 2003). The same may be true in moss, but high levels of PpSGT1 has a negative effect on AFB levels and auxin signaling.

The effect of auxin, ABA, and high temperature on *PpSGT1* expression

In tobacco *NtSGT1* is induced by *potato virus X* viral movement protein TGBp3 (Ye et al. 2012) while in wheat, the gene is induced by fungal pathogens (Xing et al. 2013). Arabidopsis has two *SGT1* genes, *SGT1a* and *SGT1b*. *SGT1a* expression is induced in stress conditions such as bacterial pathogen and high temperature, (Noël et al. 2007). SGT1a protein level is also increased in the leaves under high temperature and hydrogen peroxide treatment (Yabuta et al. 2009). However, SGT1b transcript and protein level respond marginally or remain constant in the same conditions (Noël et al. 2007). *PpSGT1* transcript level was analyzed after treatment with auxin, ABA, and high temperature (Figure 2.10A and C). Among these conditions, *PpSGT1* expression was only induced by high temperature (37℃). Other conditions did not affect *PpSGT1* transcript level changes both in the 2 hour and 24 hour treatment. It has been known that ABA signaling is linked to many stress responses including heat and pathogen stresses (Nakashima, Yamaguchi-Shinozaki, and Shinozaki 2014; Atkinson and Urwin 2012). However, *PpSGT1* expression was not induced by ABA, suggesting that stress regulation of *PpSGT1* transcription is independent of ABA signaling pathways. Under the same conditions, auxin inducible markers (*IAA1a* and *IAA1b*) were analyzed. After a 2 hour, *IAA1a* and *IAA1b* were significantly induced only by auxin (Figure 2.10B). In 24 hour-treated samples, auxin-mediated *IAA1a* and *IAA1b* transcript induction was significantly diminished (Figure 2.10D). It might be the

result of either water stress when the moss tissue samples were submerged in liquid BCD medium for 24 hours, or unknown regulatory mechanisms to offset the effects of long-term auxin treatment. In the case of high temperature treated samples, *IAA1a* and *IAA1b* transcript levels were significantly suppressed compared to the untreated condition. In *Arabidopsis*, 29° C promotes endogenous auxin level by inducing auxin biosynthesis enzyme expression, and transgenic plants harboring auxin responsive reporter *pIAA4-GUS* showed increased GUS staining in 10 day grown seedlings in high temperature condition (Gray et al. 1998; Sun et al. 2012). However, transcript levels and GUS reporter signal may not be directly compared because transcript regulation is more dynamic than auxin responsive GUS reporter signal. Besides, reduced *IAA1a* and *IAA1b* transcript levels also can be interpreted as a positive auxin response because Aux/IAAs act as repressors of ARF transcription factors. However, this result supports reduced *IAA1a* expression in *PpSGT1* overexpression lines. Although *IAA* gene transcript levels were not affected by high *PpSGT1* transcript level in 2 hour high temperature treatment, *PpSGT1* transcription induction was much stronger in the overexpression lines which caused reduced *IAA1a* transcription. Analyzing *IAA1a* transcript level by simultaneous treatment with auxin and high temperature might provide more informative results to interpret the relationship between *PpSGT1* and *Aux/IAA* levels. In addition, temperature effect on auxin responses in moss needs to be analyzed.

GDA effect on auxin response in moss

SGT1 is a co-chaperone of HSP90 and is known to be a stabilizing factor for many NLR proteins, affecting the NLR protein-mediated disease responses. Further the interaction of SGT1 and HSP90 is required for SGT1 function on HSP90 client protein stability and activation (Botër et al. 2007; Shirasu 2009). To analyze whether HSP90 function is required for auxin responses in moss, *NLS4*;*DR5:DsR* auxin responsive reporter line was treated with the HSP90 specific inhibitor geldanamycin (GDA) (Taldone, Sun, and Chiosis 2009). Upon auxin treatment, auxin responsive DsRed signal was strongly enhanced in the protonemal tissues (Figure 2.11A). However, GDA appeared to inhibit the DsRed signal induction in a concentration dependent manner. Flow cytometry analysis using protoplasts also showed that DsRed cell population in the GFP gate was increased in the 10 µM NAA treated sample (Figure 2.11B and C). As expected, GDA treatment greatly suppressed NAA-mediated reporter expression (Figure 2.11C). Thus, these results suggest that HSP90 functions as a positive component in auxin signaling in moss.

GDA effect on AFB2-myc accumulation in moss

Next, the effect of HSP90 on the auxin receptor AFB2 was analyzed. The moss line *Pubi:AFB2myc* was treated with NAA and GDA for 24 hours before immunoblot analysis. In the presence of NAA, high levels of AFB2-myc were observed compared with the untreated sample (Figure 2.12). Because

Pubi:AFB2myc expresses AFB2-myc under control of the ubiquitin promoter, this strong AFB2-myc signal is probably due to reduced AFB2-myc turnover, which suggests that auxin has inhibitory function in AFB2-myc turnover. On the other hand, AFB2-myc signal was strongly reduced in moss tissues incubated with GDA. Thus, HSP90 seems to be required for *de novo* AFB2-myc protein synthesis or maturation. In addition, it might also suggest that HSP90 might be required for AFB2-myc stabilization in the presence of auxin. *Pubi:AFB2myc* sample treated both GDA and NAA also exhibited very low AFB2-myc levels, which suggests that HSP90 function is indeed required for auxin-mediated AFB2-myc stabilization. Based on the data from *NLS4*;*DR5:DsR* and *Pubi:AFB2myc*, HSP90 is required for AFB auxin receptor synthesis and/or stabilization, and increased amount of AFBs in the presence of auxin activates downstream auxin signaling pathways. *PpSGT1* overexpression has a negative effect on AFB2-myc. AFB2-myc protein level was reduced in the *PpSGT1* overexpressing lines. Thus, PpSGT1 could assist in the stabilization or activation of some HSP90 client proteins that have a negative effect on AFB accumulation and stabilization. If PpSGT1 directly interacts with AFB proteins, it is possible that too much PpSGT1 in the cell could interfere with the interaction between AFB proteins and HSP90. One interesting observation is that auxin stabilizes AFB2-myc. It remains to be elucidated how auxin affects AFB levels. One possibility is that, in the presence of auxin, SCF^{AFB} substrates are ubiquitinated and degraded. It has been reported that in the absence of substrates, E3 ubiquitin ligases are

targeted for degradation by self ubiquitination or through external ligases (de Bie and Ciechanover 2011). According to this model AFB proteins would be degraded through an autocatalytic mechanism when auxin or Aux/IAA proteins are low. An increase in auxin levels will promote binding of the Aux/IAA substrates and thus limit autoubiquitination.

CONCLUSIONS

PpSGT seems to have important roles in moss development. No *SGT1* knock-out mutants were obtained with the two different knock-out vector constructs. Reflecting the important role of *SGT1*, *Arabidopsis SGT1* double mutant (*sgt1a sgt1b*) has an embryo lethal phenotype. One possible approach to confirm the lethality of *SGT1* mutant is to knock-in *SGT1* gene with *Cre/Lox* recombination sequences at the both end, then delete the *SGT1* insert. If the *SGT1* knock-out mutant cannot be created, the RNAi system could be used to knock-down gene expression.

SGT1 overexpression has a negative effect on auxin responses in moss. *SGT1* overexpression caused minor growth defects with similarity to some weak auxin signaling mutants. *SGT1* overexpression also suppressed the auxin hypersensitive phenotype caused by overexpression of the Arabidopsis AFB2 protein. Auxin responsive genes and an auxin reporter were suppressed by *SGT1* overexpression. However, *SGT1* overexpression did not significantly affect gene expression changes induced by auxin. Thus SGT1 seems to negatively affect upstream signaling components of auxin

response genes. To support this, *SGT1* overexpression caused reduced AFB2 accumulation. In *Arabidopsis*, *SGT1* is thought to have a positive effect on auxin responses. Thus, further detailed studies will be required to resolve this issue. In moss, high temperature induced *PpSGT1* expression. In the same conditions, auxin-responsive gene expressions were suppressed, which supports the negative effect of *PpSGT1* on auxin signaling. However, the direct links between the *PpSGT1* induction and the suppressed expression of auxin-responsive genes are still missing.

HSP90 has a positive effect on auxin signaling in moss. The HSP specific inhibitor GDA inhibited auxin reporter expression, and AFB2 accumulation was suppressed by GDA as well. There are some reports about functional decoupling between SGT1 and HSP90, but, the contrasting effects of SGT1 and HSP90 on AFB protein level cannot be explained at this point. Further studies will be needed to resolve this issue.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions and treatments

Physcomitrella patens Gransden-2004 was used as the wild type plant. Plants were grown at 25℃ under constant white light at an intensity of 40-70 μmol/m^2/second. BCD medium (1mM $MgSO_4$, 1.84mM KH_2PO_4, 10mM KNO_3, 45μM $FeSO_4$, 1mM $CaCl_2$, 1x (1mL/L) Hoagland's A-Z trace element solution) was used as a minimal culture medum. For the preparation of solid medium, 0.8% (w/v) agar was used. Diammonium tartrate (5mM; BCDAT), GDA, NAA,

ABA were supplemented into the medium where specified. For propagation, protonemal tissues were homogenized in a Waring blender under sterile condition, and spread onto solid medium covered with a cellophane film. For colony growth, small pieces of protonemal tissue (1~2 week-grown) were transferred onto a fresh BCD plates. BCD liquid medium was used to prepare samples for hormone, drug, or temperature treatment.

Moss transformation and screening of transgenic lines

To isolate protoplasts, 7 day-subcultured protonemal tissues were treated for 30 minutes with sterile 0.5% Driselase (Sigma D-9515) dissolved in 8% mannitol. Protoplast suspension was filtered through a 70 μm mesh, and pelleted twice with 8% mannitol by centrifugation at 100g for 5 minutes. Protoplasts were resuspended in MMM medium (0.4M mannitol, 15mM MgCl2, 4mM MES [pH 5.7]) at 1.5~2x10^6 cells/mL. For transformation, 20μL linearized plasmid DNA (1ug/μL) was added to 300μL protoplast suspension, and added 300 μL PEG solution (28.5% PEG 6000 in 7.2% mannitol, 0.1M $Ca(NO_2)_3$, 10mM Tris-HCl [pH8.0]). The mixture was incubated at 45℃ for 5 minutes, and diluted with 5mL of 8% mannitol solution. Then, protoplasts were pelleted by centrifugation at 100g for 5 minutes, and resuspended in 1mL of 8% mannitol solution. Before plating onto plates, 5mL of molten (45℃) agar (BCDAT, 8% mannitol, 0.4% Agar, 10mM $CaCl_2$) was added, and dispensed onto PRMB plates (BCDAT, 6% mannitol, 0.8% Agar, 10mM $CaCl_2$) covered

with cellophane film. After 5 days of incubation, regenerated protoplasts were transferred to BCDAT medium with 20 µg/ml of hygromycin for selection.

Molecular Cloning

To create *ppsgt1-2* knock-out plasmid construct, genomic DNA fragments flanking on both sides of *PpSGT1* coding region were amplified by PCR, and cloned into *pENTR-D/TOPO* (Invitrogen). These fragments were cloned into hygromycin resistant cassette containing *pMP1159* vector (http://web.mac.com/mjprigge/Prigge/Moss.html). The primer sequences used to amplify the upstream flanking sequences (1326bp) were PpSGT1uF (5'-AAGTCGACTAGAGCGTCAATTGAAGATTTTCACTC-3') and PpSGT1uR (5'-AAGGATCCGATGGCCCAAGACAATACTCCTC-3'). *SalI* and *BamHI* restriction sites were used to insert the fragment into the *SalI* and *BamHI* digested pMP1159. The downstream flanking sequnces (1000bp) were amplified with PpSGT1dF (5'-AAGCTAGCTGCAATCCTGATTAGCTCAAGC-3') and PpSGT1dR (5'-AATTTAATTAAGGTATCAGCGAATCACTTTGACTC-3'). *NheI* and *PacI* restriction sites were used to clone the fragment into *SpeI* and *PacI* digested 1159 vector backbone. *SalI* and *PacI* site were used to obtain the linearized vector for transformation. To construct *PpSGT1* overexpression construct, PpSGT1 cDNA cloned into *pENTR* plasmid was transferred into pTHUBIGate vector with LR Clonase enzyme (Invitrogen) (Vidali et al. 2007). *SwaI* restriction sites were used to linearize *PpSGT1* expression cassette for transformation.

Quantitative RT-PCR

Total RNA was isolated from protonemal tissue using the TRI-Reagent (Sigma-Aldrich), and treated with DNaseI using the DNA-free Kit (Ambion). 500µg of total RNA was used for cDNA synthesis with the GoScript Reverse Transcription System (Promega) using oligo(dT) primer. Each 20µL RT reaction was diluted with 80µL of water. PCR samples contained 2 µl diluted cDNA, 200 µM each dNTP, 250 nM each primer, 1X SYBR-Green (Invitrogen), 5% DMSO, 0.1% Tween-20, 200 ng BSA, 1.5mM MgCl2, 50mM KCl, 10mM Tris-HCl pH 8.5, and 0.2µl Taq DNA Polymerase. The normalized expression (ΔΔCt method) were calculated using Bio-Rad CFX manager system with the reference gene PpACT. Following primers were used: PpSGT1-F: 5'-TCAGGGCAGAAGAGTCAAAGGC-3', PpSGT1-R: 5'-TCGGCACTGATTCTTGGGTACG-3', PpIAA1A-F: 5'-ATCCGGGAGTCCGAGCTTC-3', PpIAA1A-R: 5'-GGTTCTGCGCAGGAGGTG-3', PpIAA1B-F: 5'-CGGTGGTCAGAATGGGTCA-3', PpIAA1B-R: 5'-CCCACAGTCTGGTTCTGCG-3', PpACT-F: 5'-CAGCCTTTGGTGTGCGACAA, PpACT-R: 5'-ACATACGCGTCCTTCTGTCC-3'. Experiments with hypocotyl or seedling tissue were done with two biological replicates and three technical replicates.

Protein immunoblot analysis

Total plant protein for western-blot analysis was extracted from protonemal tissue in 50mM Tris pH 7.5, 150mM NaCl, 10% glycerol, 0.1% NP40 and Complete EDTA-free Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). 20 µg of protein samples were analyzed using SDS-PAGE. The equal protein loading was shown by Coomassie blue or Ponceau S staining. AFB2-myc protein was detected with an HRP-conjugated anti-c-Myc antibody (Roche), or SGT1 antibody was raised in rabbit. Anti-rabbit IgG (POD) antibody produced in goat (SIGMA) was used as a secondary antibody.

Phylogenetic analysis

Protein sequences were obained from NCBI (www.ncbi.nlm.nih.gov/) and phytozome website (http://www.phytozome.net/). Amino acid sequences of Arabidopsis SGT1b were used to obtain homologous sequences from the databases using BLAST program (BLASTP and TBLASTN). Phytozome Identification number for the protein sequences as follows: *Oryza sativa* (Os01g43540.1), *Physcomitrella patens*(Pp1s244_58V6.1), *Selaginella moellendorfii* (266703), *Volvox carteri* (Vocar20014167m), *Chlamydomonas reinhardtii* (Cre12.g513600.t1.2), *Zea mays* (GRMZM2G149704_T02, GRMZM2G105019_T01), Sorghum bicolor (Sb03g028430.1), *Solanum tuberosum* (PGSC0003DMP400036282, PGSC0003DMP400050363), *Solanum lycopersicum* (Solyc03g007670.2.1), *Vitis vinifera* (GSVIVT01028818001), *Citrus sinensis* (orange1.1g015029m), *Gossypium raimondii* (Gorai.008G273400.1, Gorai.003G069500.1, Gorai.004G116900.1),

Brassica rapa (Bra000741, Bra013710, Bra035239, Bra019295), *Prunus persica* (ppa007614m), *Cucumis sativus* (Cucsa.108260.1), *Medicago truncatula* (Medtr5g009930.1), *Glycine max* (Glyma11g02341.1, Glyma01g43150.1), *Populus trichocarpa* (Potri.004G071100.1, Potri.017G149800.1), *Ricinus communis* (30190.m011280, 30169.m006578), *Capsella rubella* (Carubv10001261m, Carubv10005128m), *Aquilegia coerulea* (Aquca_013_00188.1), *Eucalyptus grandis* (Eucgr.A02714.1), *Carica papaya* (evm.model.supercontig_107.130). Yeast *S.cerevisiae* SGT1 (YOR057W) was used as an outgroup. Phylogenetic tree was constructed with maximum likelihood (ML) analysis using PhyML program with Jones-Taylor-Thornton (JTT) substitution model for amino acids substitution (Jones, Taylor, and Thornton 1992). The substitution model was determined based on ProtTest program (Abascal, Zardoya, and Posada 2005). The tree was drawn using FigTree program (http://tree.bio.ed.ac.uk/software/figtree/).

Flow Cytometry

Protoplasts were isolated from protonemal tissues (7 days after subculture) from WT and NLS4;DR5:DsR. Protonemal tissues grown for 5 days or 6 days were submerged in BCD liquid medium until protoplasting. NAA or GDA were added to the media when they are needed. Emission was measured at 530/30 nm for GFP and 610/20 nm for DsRed after excitation by a 488 nm laser using LSR-II Flow cytometer (BD Biosciences). Relative

DsRed expression levels were measured based on their red-to-green fluorescence ratio.

ACKNOWLEDGEMENTS

Chapter 2 consists of work I have undertaken during my thesis research. Mark Estelle directed and supervised the research described in this chapter.

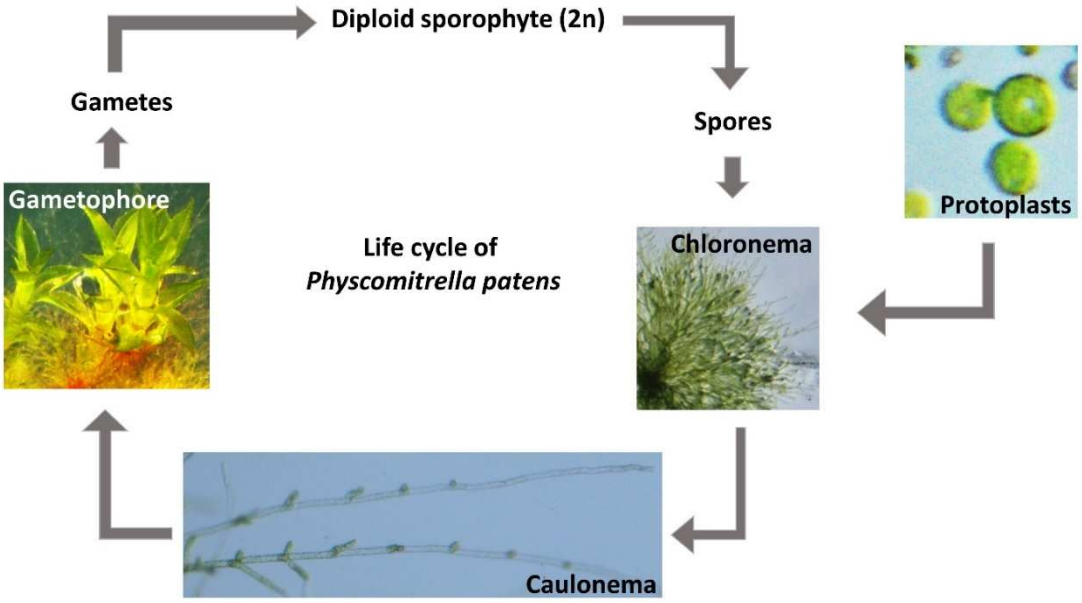

Figure 2.1 Life cycle of *Physcomitrella patens*. A haploid spore or a protoplast germinates and forms protonemal filaments, which is composed of two types of cells. The chloroplast-rich chloronemal filaments develop first from the germinating spores, and the caulonemal filaments are differentiated from the chloronemal cells. Later in the life cycle, some side branch initials develop into bud, which give rise to gametophores with shoots, leaflets, and rhizoids. As a monoicious moss, *Physcomitrella patens* develops both male and female reproductive organs at the tip of the gametophores in the same plant. Male and female gametes produced from the mature gametophophytes fuse to give rise to the zygote. The resulting diploid zygote develops into the sporophyte, which produces haploid spores by meiosis.

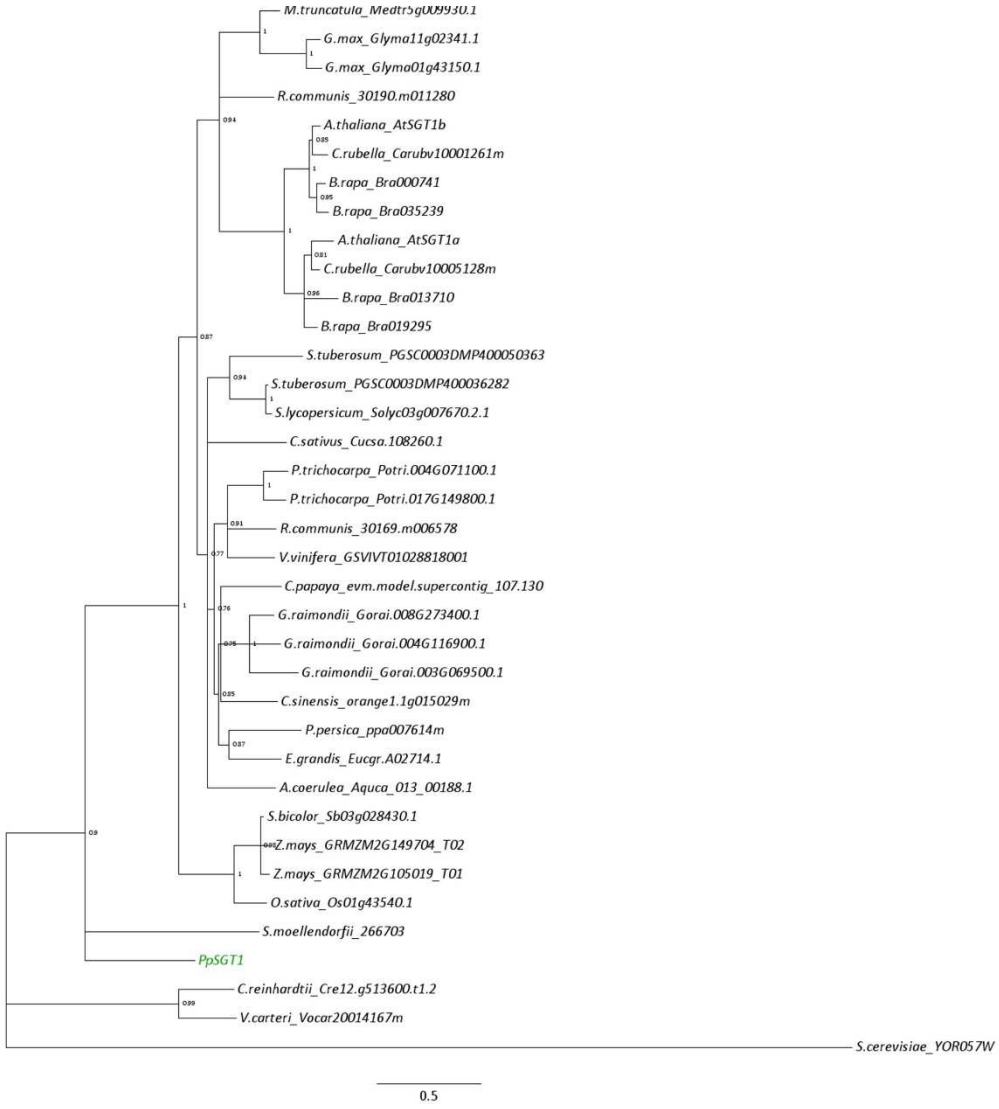

Figure 2.2 Phylogenetic analysis of *SGT1* genes. Maximum-likelihood tree of deduced SGT1 protein sequences of 24 plants and the budding yeast. The tree was generated by phyML program. Branch support values (aRLT) were displayed, and branches with aRLT values below 0.75 were collapsed.

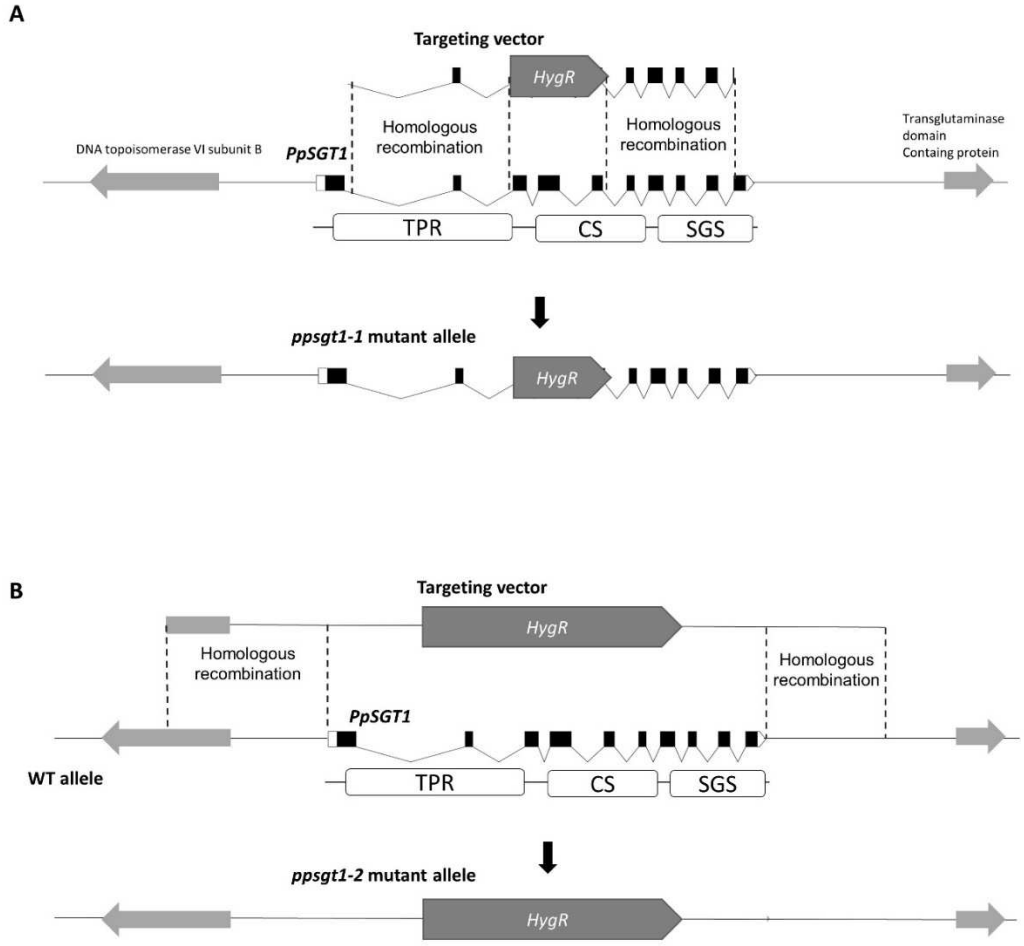

Figure 2.3 Schematic diagram of *PpSGT1* gene replacement by homologous recombination. Strategy of targeted disruption of *PpSGT1* locus by (A) removing exon 3, 4, and 5 which encode part of conserved CS domain or (B) removing the entire genomic region of *PpSGT1*.

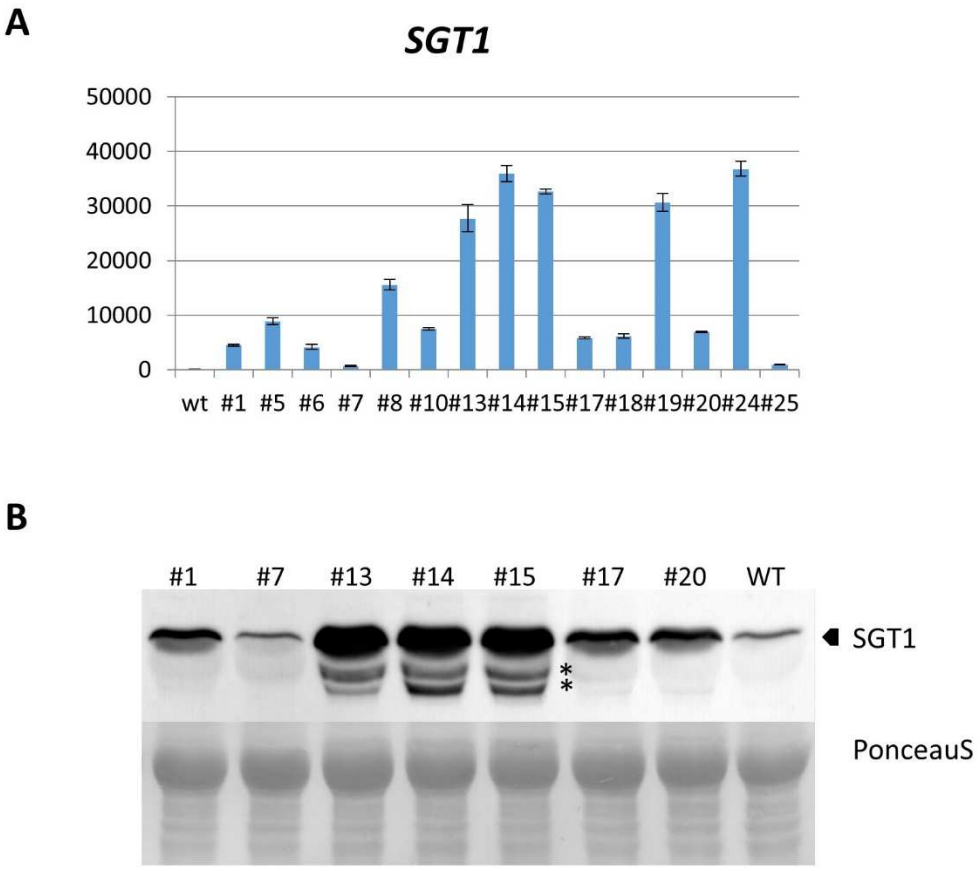

Figure 2.4 *SGT1* overexpression lines. (A) Relative expression of *SGT1* in moss WT and *SGT1* overexpressing moss plants grown on BCDAT agar medium for 8 days. The *SGT1* level was normalized to moss *actin* gene. N=3, error bars = SD. (B) Western blot analysis of total protein extracts from WT and SGT1 overexpressing transgenic lines. Degradation products were indicated (*).

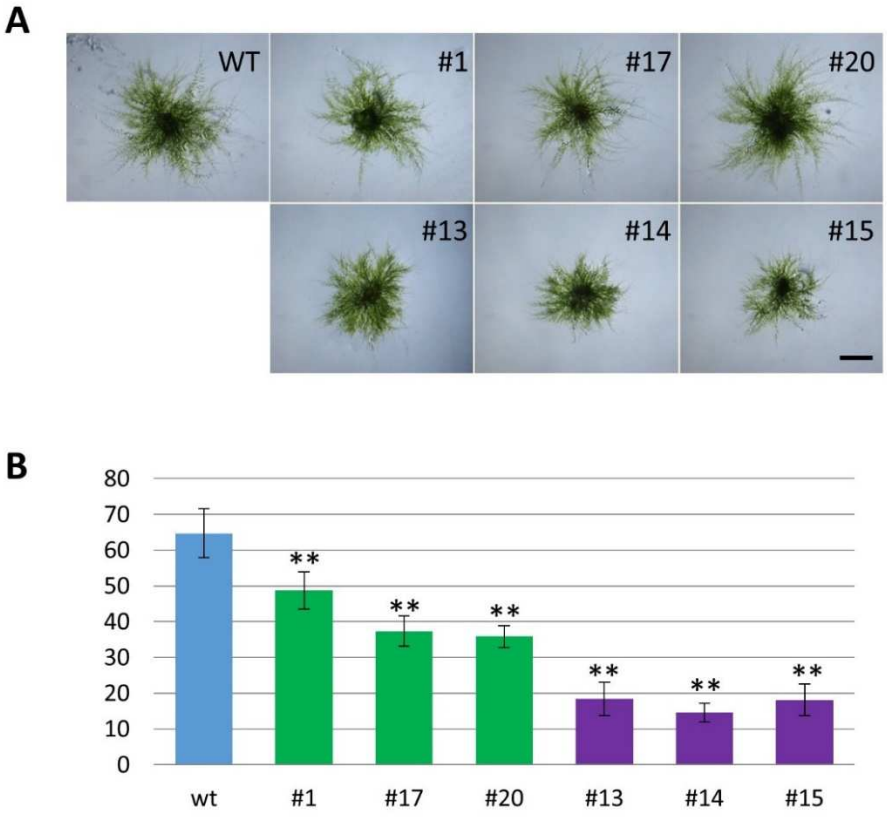

Figure 2.5 Colony phenotypes of *SGT1* overexpression lines. (A) Colony phenotype of WT and SGT1 overexpressing moss plants. Plants were grown on BCD medium for 14 days under continuous light at 24°C. (B) The number of gametophores per colony grown for 27 days on BCD medium. N=8, error bars = SD, ** $P < 0.01$ (Student t-test). The scale bar in (A): 3mm.

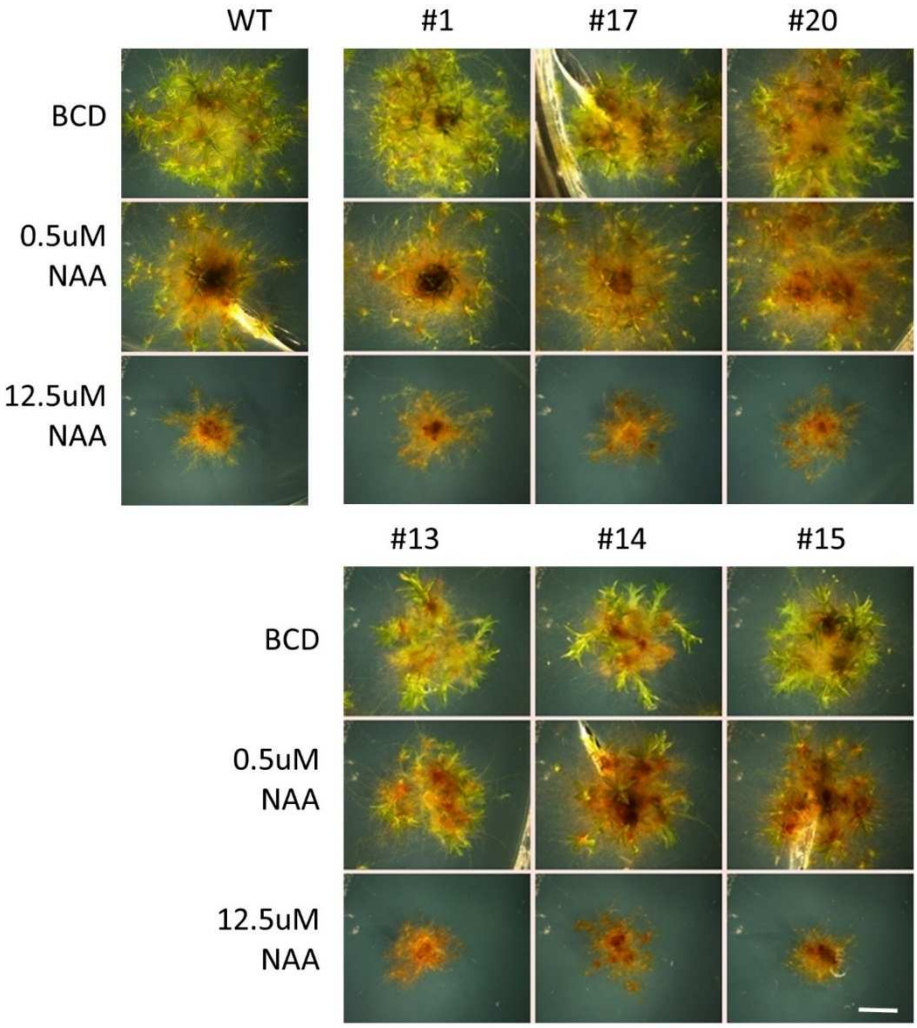

Figure 2.6 Auxin responses of WT and *SGT1* overexpressing lines. WT and SGT1 overexpressing moss plants were grown on BCD, BCD with 0.5μM NAA, or BCD with 12.5μM for one month under continuous light at 24°C. The scale bar: 5mm.

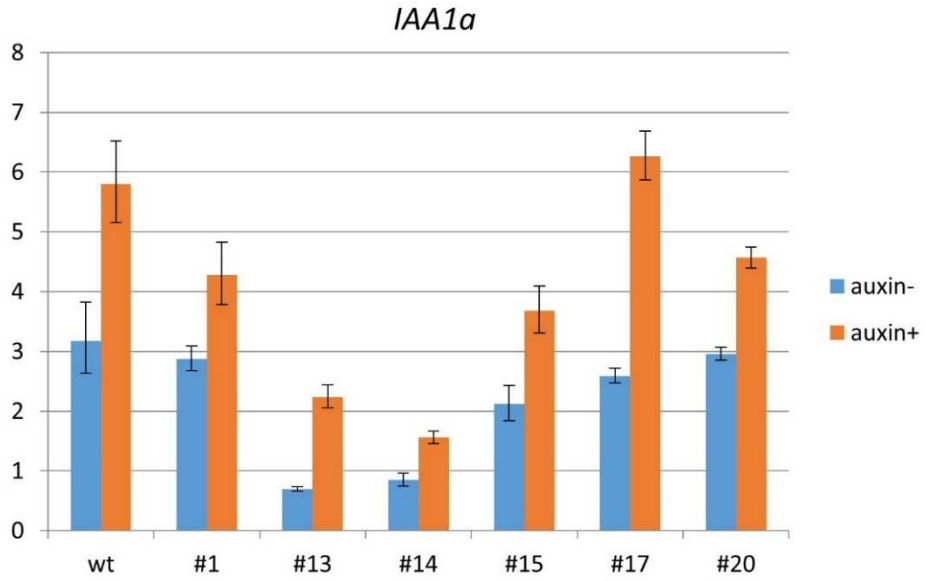

Figure 2.7 Auxin responsive gene expression in moss lines overexpressing *SGT1*. Relative expression of moss *IAA1a* in moss WT and *SGT1* overexpressing moss plants grown on BCDAT agar medium for 8 days. Moss tissues were submerged in liquid BCD medium with or without 12.5µM NAA for 2 hours. The expression level was normalized to the moss *actin* gene. N=3, error bars = SD.

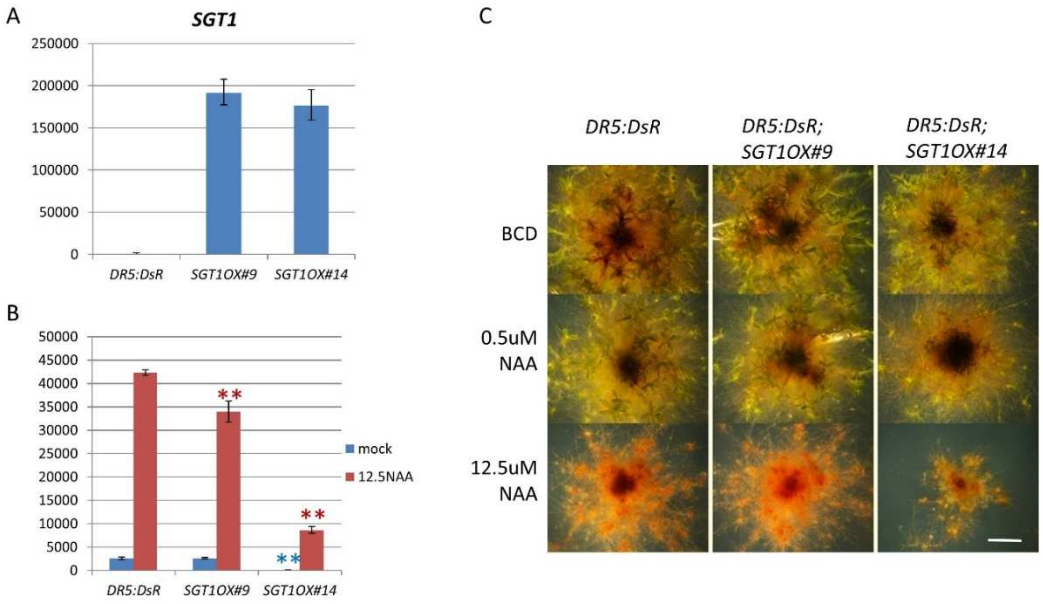

Figure 2.8 The effect of *SGT1* overexpression on auxin reporter expression in moss. (A) *SGT1* expression levels in 7 day-old moss NLS4;*DR5:DsR* and *SGT1* overexpressing lines in the NLS4;*DR5:DsR* background. N=3, error bars = SD. (B) *DsRed* reporter expression assays with protoplasts from *SGT1* overexpressing lines in the NLS4;*DR5:DsR* background. 5-day-old moss tissue samples were submerged in BCD liquid medium with or without 12.5µM of NAA for 48 hours before protoplast analysis using flow cytometry. N=3, error bars = SD. Asterisks in blue indicate a statistically significant difference compared to mock-treated samples according to a Student's t-test (** $p<0.01$), asterisks in red is for NAA-treated samples. (C) Colony growth responses to auxin. NLS4;*DR5:DsR* and *SGT1* overexpressing lines in the NLS4;*DR5:DsR* background were grown on BCD medium with or without NAA for one month under continuous light at 24°C. The scale bar in (C): 5mm.

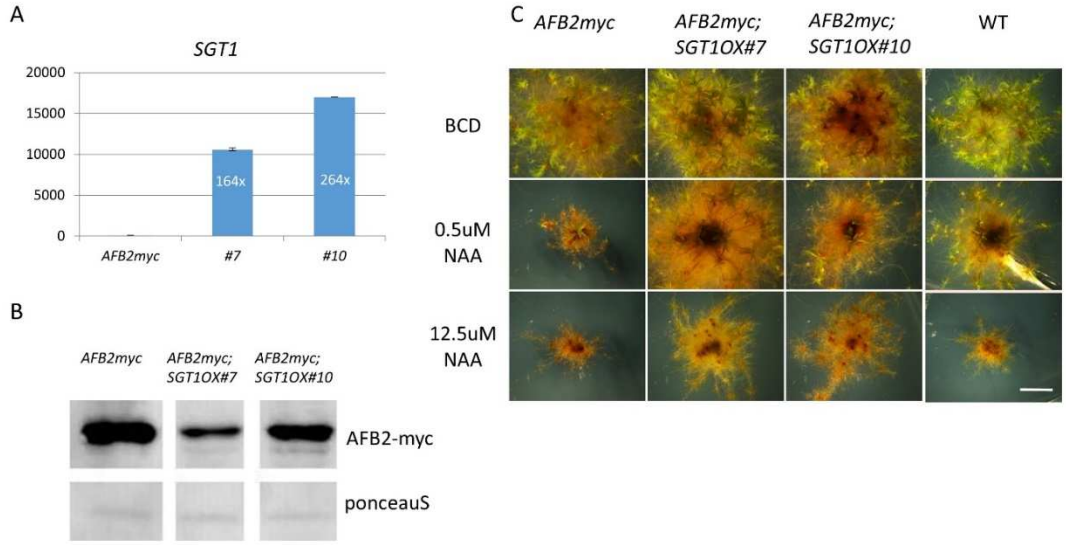

Figure 2.9 The effect of *SGT1* overexpression on AFB2 level in moss. (A) *SGT1* expression levels in 7-day-old moss *Pubi:AFB2myc* and *SGT1* overexpressing lines in the *Pubi:AFB2myc* background. N=3, error bars = SD. (B) AFB2myc protein levels in *Pubi:AFB2myc* and *SGT1* overexpressing lines in the *Pubi:AFB2myc* background. Total protein was extracted from 7-day-old moss tissue samples. AFB2 protein was detected with a c-Myc antibody. (C) Colony growth responses to auxin. *Pubi:AFB2myc* and *SGT1* overexpressing lines in the *Pubi:AFB2myc* background were grown on BCD medium with or without NAA for one month under continuous light at 24°C. The scale bar in (C): 5mm.

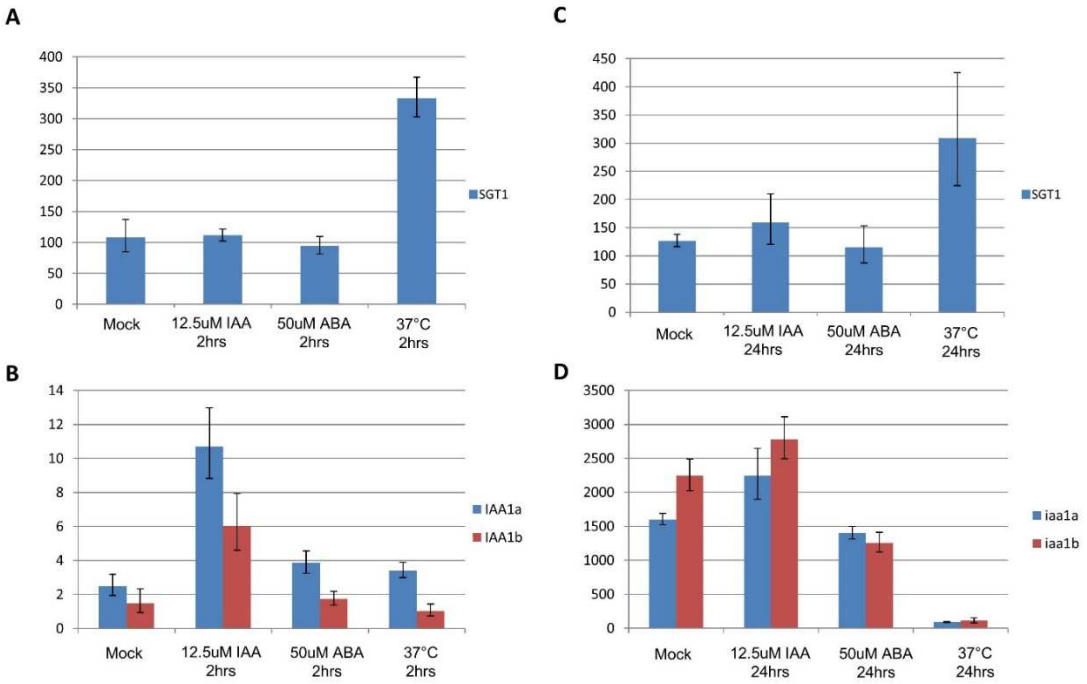

Figure 2.10 *PpSGT1* expression changes in response to IAA, ABA, or high temperature and subcellular localization. (A and C) *SGT1* expression level in response to IAA, ABA, or high temperature treatment for 2 hours or 24 hours respectively. (B and D) Expression of auxin-responsive genes *IAA1a* and *IAA1b* in response to IAA, ABA, or high temperature for 2 hours or 24 hours. WT and *SGT1* overexpressing moss plants grown on BCDAT agar medium for 8 days. For hormone and high temperature treatment, liquid BCD medium was used. The expression level was normalized to moss *actin* gene. N=3, error bars = SD.

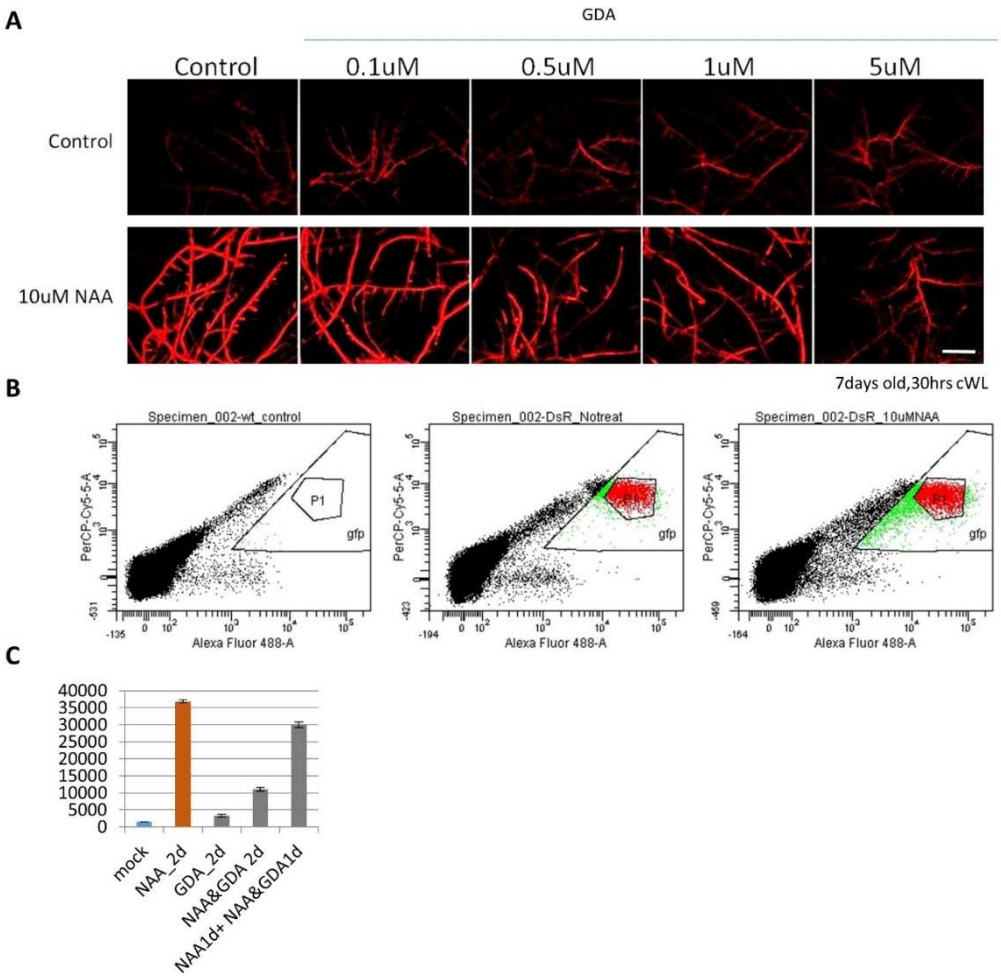

Figure 2.11 The effect of GDA on auxin reporter expression in NLS4;*DR5:DsR*. (A) DsR expression pattern in moss colonies in different concentrations of GDA and NAA. (B) Protoplasts from WT and NLS4;*DR5:DsR* were sorted with a LSRII (BD) using gates defined on a dotplot of green (530/30 nm; x axis) versus red (610/20 nm; y axis) fluorescence (100,000 events). From left to right: WT, Untreated NLS4;*DR5:DsR*, and 10µM NAA-treated NLS4;*DR5:DsR*. (C) *DsR* reporter expressions of NLS4;*DR5:DsR* protoplasts in the absence of or presence of 10µM NAA, 5µM GDA, 10µM NAA + 5µM GDA for 2 days, or 1 day of 10µM NAA pretreatment followed by 10µM NAA + 5µM GDA treatment. BCD liquid medium was used for the treatments. N=3, error bars = SD. The scale bar in (A): 200µm.

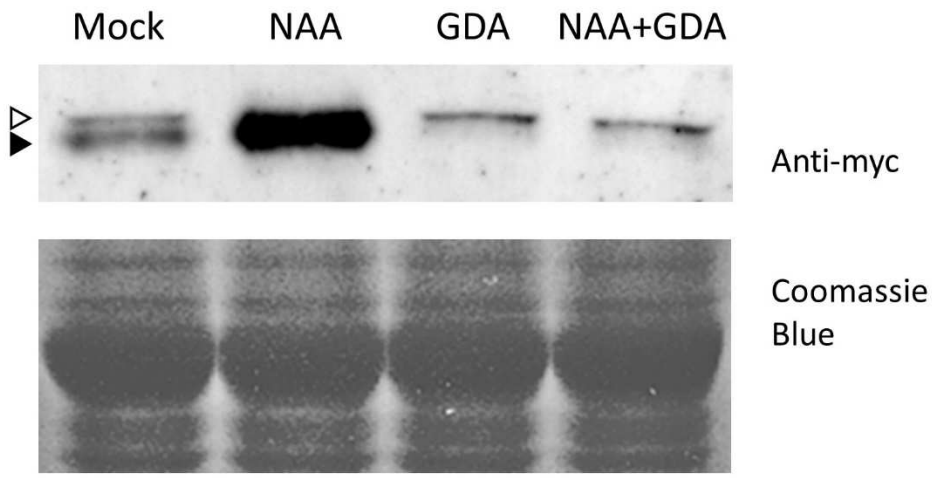

Figure 2.12 The effect of GDA on AFB2-myc protein accumulation in moss. Immunoblot analysis of moss *Pubi:AFB2myc* treated with 10µM NAA or 5µM GDA. AFB2-myc was detected with a c-Myc antibody. For treatment, 7 day-old *Pubi:AFB2myc* tissue samples were submerged in BCD liquid medium with or without NAA or GDA for 24 hours.

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