

GEMINIVIRUS C4 ONCOPROTEIN INTERACTS WITH AND IS ACTIVATED BY
ARABIDOPSIS SHAGGY-LIKE PROTEIN KINASES LEADING TO DISRUPTION OF
BRASSINOSTEROID SIGNALING AND ALTERED ARABIDOPSIS DEVELOPMENT

by

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(Under the Direction of Carl M. Deom)

ABSTRACT

The C4 protein of *Beet curly top virus* (BCTV-B [US:Log:76]) induces hyperplasia in infected phloem tissue and tumorigenic growths in transgenic plants. The protein offers an excellent model for studying cell cycle control, cell differentiation and plant development. Expression of the C4 transgene, under an inducible promoter, in *Arabidopsis thaliana* plants (line IPC4-28) lead to extensive cell division in all tissues types examined, and radically altered tissue layer organization and organ development. Induced seedlings failed to develop shoot and root meristems, vascular tissue, true leaves and lateral roots. Exogenous application of brassinosteroid and abscisic acid weakly rescued the C4-induced phenotype, while induced seedlings were hypersensitive to gibberellic acid and kinetin.

The C4 protein interacts *in planta* with three closely related members of the Arabidopsis SHAGGY-like protein kinase (AtSK21, AtSK22 and AtSK23) family, which are negative regulators of brassinosteroid (BR) hormone signaling. AtSK21 is known to hyperphosphorylate BES1 and BZR1 transcription factors, thereby inhibiting their function. The expression of C4 in IPC4-28 plants resulted in dephosphorylation of the BES1 transcription factor and changes in the

expression levels of BES1 and BZR1 target genes. These changes were similar to those that occur when AtSK21 is inactivated by BR hormone treatment. Using several approaches a putative phosphorylation site (Ser at residue 49) on C4 is shown to be required for the C4/AtSK interaction. Mutation of Ser49 to Ala abolishes the C4/AtSK interaction, loss of the C4-expression phenotype, and failure to induce changes in the BR signaling pathway. Substitution of Ser49 by Thr restores both the C4/AtSK interaction and the effects of C4 on plant development and BR signaling. An active kinase is also required for the interaction, as AtSK kinase dead mutants fail to interact with C4. Taken together this indicates that C4 is a substrate for AtSK kinase activity and functions, at least in part, through disruption of the BR signaling pathway.

INDEX WORDS: Geminivirus, Beet curly top virus, C4 gene, oncogene, brassinosteroid, Arabidopsis SHAGGY-like protein kinase, phosphorylation, development

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DEDICATION

To my grandfather, Carlos Luján, you will always be with me.

To my husband Canek Fuentes, you danced into my life and have led me ever so caringly across the room into a whole new world of opportunities. The world is our dance-floor and they're playing our song.

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CHAPTER 1

INTRODUCTION

Geminiviridae, a family of circular single stranded DNA viruses, are important pathogens on crops worldwide (Varma and Malathi, 2003; Seal et al., 2006), as well as important models for studying host-pathogen interactions (Hanley-Bowdoin et al., 1999; Lazarowitz, 1999; Voinnet, 2001; Gutierrez, 2002). All plant viruses are dependent on their host for replication, and geminiviruses are no exception. In fact, unlike RNA viruses, geminiviruses do not encode a polymerase. This makes geminiviruses unique in their requirement for the host cell's DNA replication machinery to be active (Hanley-Bowdoin et al., 1999; Hanley-Bowdoin et al., 2004), following transport of the viral genome to the nucleus for replication. Because geminiviruses are excluded from meristematic tissues (Peele et al., 2001), which are actively dividing, and instead infect mostly terminally differentiated cells (Rushing et al., 1987; Nagar et al., 1995; Morra and Petty, 2000), they must shift non-dividing cells from a quiescent state into S-phase or the DNA replication phase of the cell cycle (Hanley-Bowdoin et al., 1999). Some geminiviruses shift cells into endoreduplication (Bass et al., 2000; Hanley-Bowdoin et al., 2004), bypassing the need for cell division. However, many geminiviruses induce cell division during infection (Latham et al., 1997; Briddon and Markham, 2000; Hanley-Bowdoin et al., 2004; Desvoyes et al., 2006). Such is the case for *Beet curly top virus* (BCTV), which causes enations (tumor-like growths) on the abaxial leaf surface of infected plants, leading to the formation of characteristic upward leaf curling (Esau and Hoefert, 1978; Park et al., 2004). The C4 gene encoded by BCTV has been

found necessary and sufficient for causing cell division in plants (Stanley and Latham, 1992; Latham et al., 1997). However, an exhaustive characterization of the effects of C4 on plants has not been done, nor is the mechanism behind the function of C4 known; both of which will be the focus of this dissertation.

BCTV was chosen as a model for the study of C4 because, in addition to the distinct cell division induced on its host plants, BCTV infects the widely used model plants *Arabidopsis thaliana* and *Nicotiana benthamiana*, and crop plants such as sugarbeet (*Beta vulgaris* L.) and spinach (*Spinacia oleracea* L.; Esau and Hoefert, 1978; Matthews, 1991). *Arabidopsis* is an important model plant, and as such presents many favorable characteristics, including a short life cycle, it produces many seeds by autopollination, it can be transformed easily, the complete genome has been sequenced and is publicly available, and there are many molecular tools available for its study (Meyerowitz, 2001). The *A. thaliana* ecotype Sei-0 was chosen because it is more susceptible to BCTV, and viral DNA accumulation is higher than in *A. thaliana* ecotypes Col-0, Ws-0, or other less commonly used ecotypes (Lee et al., 1994). Similarly, *N. benthamiana* has been used extensively to study plant viruses because of its enhanced susceptibility to many viral species, and the availability of tools for studying protein-protein interactions and protein localization *in planta* (Goodin et al., 2008). An additional advantage to using BCTV is that it has two close relatives, *Beet curly top severe virus* (BSCTV) and *Beet mild curly top virus* (BMCTV) that show differences in infection symptom severity (Lee et al., 1994; Stenger, 1998), which could potentially be linked to differences in C4 function. Therefore, comparison of the C4 genes of these three viruses could help gain further insight into the necessary C4/host protein interactions required for induction of cell division.

Using BCTV C4 and both *A. thaliana* and *N. benthamiana*, as host-pathogen interaction systems, this research focused on two main objectives:

- 1) A detailed phenotypic characterization of the effects of C4 protein expression on plant development
- 2) Determination of the mechanism behind the function of C4

The third chapter in this dissertation (Mills-Lujan and Deom, 2010) addresses the first major objective. Transgenic *A. thaliana*, ecotype Sei-0, expressing the BCTV C4 gene was used to characterize the effects of C4 expression on plant development. The effects of C4 on early and later stages of *A. thaliana* development were characterized using several microscopy approaches. It was noted that C4 affects meristem development, tissue organization and cell differentiation, in addition to increasing cell division. Preliminary experiments also indicated that hormone signaling, especially of brassinosteroids (BRs), was altered in C4-expressing plants. Further evidence for disruption of the BR signaling pathway by C4 is given in chapter four. The C4 protein was shown to interact specifically with a subset of Arabidopsis SHAGGY-like protein kinases (AtSKs), which are involved in the BR signaling response cascade *in planta*. Additionally, amino acids important for the C4/AtSK interaction were identified. C4 mutants that failed to interact with AtSKs were also shown to be non-functional in plants. The results from chapter four will be submitted for publication shortly. Chapter five contains initial studies and suggested future experiments to compare the function of BCTV C4 and BMCTV C4. Finally, chapter two contains a comprehensive literature review encompassing all chapters of this dissertation, while chapter six draws the major and overarching conclusions of the results presented herein.

A better understanding of the function of C4 could impact three different knowledge areas. First, understanding how C4 induces cell division could be harnessed to engineer crops with increased biomass, and applied in crops designated for biofuel or food production. Second, geminiviruses are important plant pathogens that cause significant economic and yield losses, and even though several approaches have been taken to decrease the incidence of geminivirus infections in crops, such as controlling insect vector populations (Lapidot and Friedmann, 2002), breeding for virus resistant crops (Pico et al., 1996; Lapidot and Friedmann, 2002), engineering transgenic crops resistant towards virus infections (Day et al., 1991; Bejarano and Lichtenstein, 1994; Hong and Stanley, 1996; Noris et al., 1996; Bendahmane and Gronenborn, 1997; Duan et al., 1997b; Aragao et al., 1998; Sinisterra et al., 1999; Hou et al., 2000), only limited success has been achieved. Thus, a better understanding of geminivirus infection is necessary for designing better control strategies. Third, geminivirus C4 protein could be used as a tool for gaining knowledge into cell- cycle regulation in plants. Unlike animals, plants normally do not develop tumors unless they are under pathogen attack (Doonan and Hunt, 1996), suggesting that cell-cycle regulation differs in plants and animals. However, there are parallels in the requirement for neovascularization in tumor formation between plants and animals (Ullrich and Aloni, 2000). Understanding how plants regulate cell-cycle progression is important, because it may provide insight into the differences between cell-cycle regulation in plants and animals, and could ultimately provide an explanation for why plants don't normally develop tumors, whereas animals do.

CHAPTER 2

LITERATURE REVIEW

The family Geminiviridae, the second largest family of plant viruses, causes huge economic and yield losses in many crops worldwide (Varma and Malathi, 2003; Seal et al., 2006). Geminivirus disease outbreaks have been attributed to intensification of agricultural practices, an increase in vector population, and emergence of new geminivirus species (Polston and Anderson, 1997; Varma and Malathi, 2003; Seal et al., 2006). Approaches used to decrease the incidence of geminivirus infections in crops include controlling whitefly populations (Lapidot and Friedmann, 2002), breeding for natural resistance (Pico et al., 1996; Lapidot and Friedmann, 2002; Seal et al., 2006) and transgenic approaches to resistance (Day et al., 1991; Bejarano and Lichtenstein, 1994; Hong and Stanley, 1996; Noris et al., 1996; Bendahmane and Gronenborn, 1997; Duan et al., 1997b; Duan et al., 1997a; Aragao et al., 1998; Sinisterra et al., 1999; Hou et al., 2000). Unfortunately, only limited success has been achieved through these approaches, making it evident that further knowledge on the infection cycle of geminiviruses is needed in order to address the issue of adequate virus disease management.

In addition to their importance as plant pathogens, geminiviruses have become increasingly popular as tools for understanding endogenous plant processes, such as cell-to-cell communication and transport of macromolecules (Lazarowitz, 1999), DNA replication and cell-cycle control (Hanley-Bowdoin et al., 1999; Gutierrez, 2002), and host-cell defense mechanisms such as gene silencing (Voinnet, 2001).

All geminiviruses are comprised of a circular single stranded DNA (ssDNA) genome, which can be monopartite or bipartite. Each ssDNA molecule, 2.5-3.0kb, is encapsidated singularly within a geminate or twinned icosahedral virus particle, which gives the family its name (IctvdB; Matthews, 1991). Because of the differences in genome organization and host range and vector requirements, geminiviruses have been divided into four genera: *Begomovirus*, *Curtovirus*, *Mastrevirus*, and *Topocuvirus* (Table 2-1; Fauquet et al., 2003; Fauquet and Stanley, 2003; Varma and Malathi, 2003). The best studied are the bipartite begomoviruses.

The genome of the bipartite begomoviruses is organized in two components, designated DNA-A and DNA-B (Fig. 2.1). There are five genes on the DNA-A component, one gene on the viral-sense strand (V-sense), AV1 (also known as AR1) encodes the coat protein (CP), and four genes on the complimentary-sense (C-sense) strand: AC1 (or AL1) encodes the replication associated protein (Rep), AC2 (or AL2) encodes the transcription activation protein (TrAP), AC3 (or AL3) encodes a replication enhancer (REn), and AC4 (or AL4) has no known function. There are only two genes on the DNA-B component: BV1 (or BR1) encodes a nuclear shuttle protein (NSP), and BC1 (or BL1) codes for the movement protein (MP) (Gutierrez, 1999; Varma and Malathi, 2003). The DNA-A and DNA-B components are different except for a 200nt sequence, known as the common region (CR), which is shared between them (Brown and Czosnek, 2002) and contains both a variable regulatory region required for bidirectional transcription (Rep binding site), and an invariable nononucleotide (9-nt), TAATATTAC, which contains the origin or replication (Gutierrez, 1999; Varma and Malathi, 2003). In the curto-, topocu-, and mastreviruses the common region is known as the intergenic region (IR), and has a similar organization and function (Gutierrez, 1999). The genome of the monopartite begomoviruses resembles that of DNA-A, except that it encodes a second open reading frame (ORF) on the V-

sense strand (Navot et al., 1991), which has been implicated in viral movement (Rojas et al., 2001).

The genomes of Curtoviruses consist of three ORFs on the V-sense strand (V1, V2, and V3), and four ORFs on the C-sense strand (C1, C2, C3, and C4) (Baliji et al., 2007). Genes of known function on the V-sense strand are V1 and V3, which correspond to the CP and MP, respectively. The C-sense strand encodes mainly genes involved in replication, such as Rep (C1) and REn (C3) (Gutierrez, 1999), whereas C2 encodes a host defense factor (Baliji et al., 2007). The functions of V2 and C4 have yet to be unequivocally determined; the latter of which will be investigated further in this dissertation.

Geminivirus infection, what do we know so far?

Geminivirus infection starts when the virus is introduced into the plant host by the insect vector (Gutierrez, 1999). The ssDNA is uncoated and transported into the nucleus by an unknown mechanism, which involves CP and host proteins (Liu et al., 1997; Kunik et al., 1998; Gafni and Epel, 2002). Once in the nucleus the viral DNA can be replicated primarily via a rolling-circle mechanism (RCR) (Saunders et al., 1991; Stenger et al., 1991); although some begomoviruses and the curtovirus BCTV are also able to replicate using recombination-dependent replication (Jeske et al., 2001; Preiss and Jeske, 2003). The initial step in RCR involves the conversion of the viral ssDNA, also known as the (+)-strand, into a dsDNA intermediate. The complimentary strand synthesized from the (+)-strand template is known as the (-)-strand. For most geminiviruses, initiation of the (-)-strand synthesis is RNA-primed and maps to the IR region (Saunders et al., 1992), elongation of the (-)-strand is completely dependent on host factors, which remain to be identified (Gutierrez, 1999).

Synthesis of new (+)-strand initiates within the conserved nanonucleotide (Stanley, 1995), which forms a hairpin loop that is recognized and cleaved by the endonucleolytic activity (Laufs et al., 1995a; Orozco and Hanley-Bowdoin, 1996) of the sequence-specific DNA-binding geminivirus Rep protein (Fontes et al., 1992). The free 3'-OH left by the nicking of the nanonucleotide (TAATATT↓AC, the arrow indicates the site of nicking) serves as a primer for polymerization, presumably by the host cell machinery (Accotto et al., 1993). During elongation of (+)-strand tandem copies of the unit length (+)-strand are generated (Gutierrez, 1999; Hanley-Bowdoin et al., 1999) and resolved by the cleavage/ligation activities of Rep protein (Heyraud-Nitschke et al., 1995; Laufs et al., 1995b). Other ssDNA viruses, such as the Nanoviridae also replicate via a RCR mechanism (Gronenborn, 2004), but the synthesis of the dsDNA intermediate form is primed by a short DNA sequence encapsidated within the viral particle (Hafner et al., 1997).

Cell-to-cell movement is necessary for a virus to move out of the initial site of infection, reach the phloem, and spread systemically. This poses a unique challenge for plant viruses because they must first overcome the cell-wall barrier (Lazarowitz and Beachy, 1999). Plasmodesmata (PD) are cylindrical cytoplasmic channels that traverse plant cell walls, thereby connecting adjacent cells. A continuity of plasma membrane and endoplasmic reticulum make up the outer and central lining of the channel, respectively, leaving an intervening cytoplasmic space in between the two membranes. Proteins studded along the membranes help regulate the passage of macromolecules from cell-to-cell (Lucas and Lee, 2004). Endogenous proteins and ribonucleoprotein complexes are trafficked through PD to exert developmental control in a non-cell-autonomous fashion (Lucas et al., 1993; Zambryski and Crawford, 2000; Nakajima et al., 2001; Lee et al., 2003; Lucas and Lee, 2004). Plant viruses have taken advantage of PD

trafficking and evolved, or acquired, the capacity to move their infectious nucleic acids cell to cell through PDs (Deom et al., 1992; Carrington et al., 1996; Lazarowitz and Beachy, 1999). In the case of geminiviruses, several proteins have been implicated in cell-to-cell and long-distance movement. Movement for bipartite begomoviruses involves the action of NSP, MP, and CP. NSP binds strongly to ssDNA, and localizes to the cell nucleus (Pascal et al., 1994), but relocalizes to the plasma membrane in the presence of MP (Noueiry et al., 1994; Sanderfoot and Lazarowitz, 1995). The CP is able to form virions, but additionally it can bind newly replicated ssDNA without encapsidating it (Qin et al., 1998), which is important for movement. In the current model NSP binds to oligomerized *A. thaliana* NSP-interactor (AtNSI) and recruits it to ssDNA-CP complexes in the nucleus. Once this ternary complex is formed, AtNSI acetylates CP (McGarry et al., 2003), reducing CPs affinity for the viral genomic DNA, allowing NSP to displace CP and export the ssDNA out of the nucleus (Carvalho et al., 2006), where it binds MP and moves as a complex from cell to cell (Gafni and Epel, 2002). For monopartite geminiviruses it appears that CP can function in trafficking the viral DNA from the nucleus to the cytoplasm, since the CP of TYLCV has both a nuclear localization signal (Kunik et al., 1998) and an active nuclear export signal (NES) (Rhee et al., 2000). However, the mechanism is still not understood and the identity of the cell-to-cell MP for monopartite geminiviruses remains unknown (Gafni and Epel, 2002).

Geminiviruses and cell-cycle regulation

In contrast to most RNA viruses, geminiviruses do not encode a *bona fide* replication protein, in contrast to most RNA viruses, and therefore are dependent on the host replication machinery for DNA polymerization and replication. The only actively dividing cells in plants are

meristematic cells (Scolfield and Murray, 2006). Once cells have exited the meristematic region they differentiate into specific cell types, depending on the tissue, and enter a quiescent phase (De Veylder et al., 2002). Cells that have already differentiated contain undetectable levels of DNA replicating enzymes (Coello et al., 1992; Nakashima et al., 1998), which poses a problem for geminiviruses since most are excluded from meristematic tissues and are found in differentiated tissues (Rushing et al., 1987; Nagar et al., 1995; Lucy et al., 1996; Peele et al., 2001). Although some geminiviruses are restricted to the vascular tissue (Horns and Jeske, 1991; Sanderfoot and Lazarowitz, 1996; Wang et al., 1996; Morra and Petty, 2000), where they could potentially use the meristematic pro-cambial cells for replication (Kong et al., 2000). Therefore, geminiviruses must either infect actively replicating cells, or shift differentiated, non-dividing cells into S-phase (see below for stages in the cell cycle) where DNA replication does occur.

The cell cycle involves four different phases: G1 (gap 1), S (DNA replication), G2, and M (mitosis). The G1/S transition is one of two main regulatory points, since it involves commitment not only to initiate DNA replication (i.e. enter S-phase), but also to complete the full cell cycle (Dewitte and Murray, 2003). Although there is evidence that some geminivirus infections can induce cells to enter into an endocycle, in which DNA is replicated in the absence of mitosis, and cells cycle between G1 and S phases only (Hanley-Bowdoin et al., 2004). The second major regulatory point in a normal cell-cycle is the G2/M boundary (Dewitte and Murray, 2003). The basic machinery that regulates the cell cycle is composed of cyclins, cyclin-dependent kinases (CDKs), CDK-activating kinases (CAKs), cyclin-dependent kinase inhibitors (CKIs), and components of the 26S proteasome (Dewitte and Murray, 2003; Inze, 2005; Inze and De Veylder, 2006). The principal regulatory proteins in the cell cycle are the CDKs, which are serine/threonine protein kinases, and are classified into six groups in plants (CDKA-CDKF)

(Joubes et al., 2000; Vandepoele et al., 2002). All eukaryotic organisms encode at least one CDK in their genome. CDKs possess a cyclin-binding domain and a catalytic CDK subunit important for substrate recognition (Inze and De Veylder, 2006). CDKs require a cyclin partner for their activity. Different CDK/cyclin complexes regulate different targets (Dewitte and Murray, 2003). In plants, CDKA plays an important role in G1/S and G2/M transitions (Inze and De Veylder, 2006). Plant-specific CDKBs are involved in the G2/M transition (Porceddu et al., 2001). CDKD and CDKF are involved in activating other CDKs, and thus are known as CDK-activating kinases (CAKs). The functions of CDKC and CDKE are still not clearly understood (Umeda et al., 2005). Plants encode a large number of cyclins, more than other organisms (Vandepoele et al., 2002). The *Arabidopsis* genome encodes 32 cyclins, ten A-type, eleven B-type, ten D-type, and one H-type cyclin (Inze and De Veylder, 2006). The D-type cyclins (CYCD) are important in the G1/S transition (Jager et al., 2005), A-type cyclins are expressed at different times of G1/S and during S-phase (Reichheld et al., 1996), and B-type cyclins are important in G2/M transition and intra-M-phase control (Jager et al., 2005). The activity of CDK/cyclin heterodimers is regulated by phosphorylation, proteolysis through the 26S proteasome, and inhibitors (Inze and De Veylder, 2006).

Similar to mammalian DNA tumor viruses, geminiviruses have been able to overcome the DNA replication barrier by interfering in host cell cycle regulation, namely by binding to the retinoblastoma (pRB) tumor suppressor protein (Herwig and Strauss, 1997; Helt and Galloway, 2003). Homologues of pRB have also been found in plants (retinoblastoma-related protein – pRBR), and they show both conservation in their functional pocket domain, and their protein-protein binding characteristics (Durfee et al., 2000; Sabelli and Larkins, 2006). Both pRB and pRBR have been implicated in regulation of cell proliferation, differentiation, and

endoreduplication (Park et al., 2005). E2F (a transcription factor) and DP (dimerization partner) form a dimer E2F/DP that transactivates the expression of genes required for G1/S transition and S-phase progression (Brehm et al., 1999; Ramirez-Parra and Gutierrez, 2000). Binding of pRB to E2F leads to disruption, and thus inactivation, of the complex (De Veylder et al., 2002). The phosphorylation of pRB by CDKs is a regulator mechanism that disrupts the interaction between pRB/E2F (Kaelin, 1999), allowing G1/S transition to proceed. In plants, pRBR is phosphorylated by the CDKA/CYCD complex (Boniotti and Gutierrez, 2001); pRBR interacts directly with the CDK/cyclin complex through the conserved LxCxE motif on CYCD (Dahl et al., 1995; Ach et al., 1997; Nakagami et al., 2002), which in turn allows CDKA to phosphorylate pRBR. Both animal viruses and ssDNA plant viruses (i.e. geminiviruses and nanoviruses) encode proteins that are capable of binding to pRB (or pRBR) (Jansen-Durr, 1996; Gronenborn, 2004; Hanley-Bowdoin et al., 2004), disrupting the pRB/E2F complex (Dewitte and Murray, 2003), and allowing cell cycle to progress. Three distinct geminivirus proteins have been found to interact with pRBR, namely RepA of mastreviruses, and Rep and REn of begomoviruses (Gutierrez, 2002). Interestingly, RepA binds to pRBR via a conserved LxCxE amino acid motif, also used by the mammalian viruses; whereas begomovirus Rep and REn do not contain the LxCxE motif, and therefore must interact with pRBR through another mechanism (Gutierrez, 2002; Hanley-Bowdoin et al., 2004).

Host plant gene expression regulation by geminiviruses appears to be different depending on genomic organization. Bipartite begomoviruses modify gene expression so that DNA replication occurs without cell division (i.e. cells enter the endocycle) (Bass et al., 2000; Hanley-Bowdoin et al., 2004). In contrast, cell proliferation is associated with the switch in cell cycle induced by curtoviruses and monopartite begomoviruses (Latham et al., 1997; Briddon and

Markham, 2000; Hanley-Bowdoin et al., 2004). Such is the case for infections of BCTV on sugarbeet, which are characterized by phloem tissue hyperplasia (Esau and Hoefert, 1978). Similarly, inoculation of *A. thaliana* ecotype Sei-0 with BCTV can cause callus-like deformations on inflorescences (Park et al., 2004), in addition to stunting and leaf curling seen on *N. benthamiana* and other ecotypes of *A. thaliana* (Lee et al., 1994). The ability of BCTV to induce hyperplasia appears to reside on the C4 gene. Early experiments noted that infections with C4 mutants of BCTV resulted in reduced pathogenicity of the virus on several hosts, but most notably the infections lacked the characteristic vein swelling (Stanley and Latham, 1992). Later it was shown that ectopic expression of C4 in *N. benthamiana* lead to the production of enations (tumorigenic growths), suggesting a role for this gene in cell division initiation (Latham et al., 1997). Similarly, disruption of the TLCV C4 is important for viral symptom development (Rigden et al., 1994) and ectopic expression of the TLCV C4 gene leads to the production of virus-like symptoms, including stunting and abnormal leaf development (Krake et al., 1998).

Current knowledge on the C4 gene

Recently it was shown that BCTV C4 interacts with AtSK21 (also known as BIN2/UCU1/DWF12/AtSK η) and AtSK23 (BIL2/AtSK ζ) in yeast two hybrid assays (Piroux et al., 2007). Additionally, AtSK21 was also able to phosphorylate C4 *in vitro* (Piroux et al., 2007). AtSK21 and AtSK23 belong to the Arabidopsis SHAGGY-like protein kinase (AtSK) family of serine/threonine protein kinases, which has ten members, divided into four subgroups (Yoo et al., 2006), although the kinase domain is highly conserved between them (Jonak and Hirt, 2002). Homologs of AtSKs, initially identified in animals (known as Glycogen synthase kinase 3), have been found in all eukaryotes examined, and are involved a variety of signaling transduction

pathways including cytokinesis, cell fate determination, metabolism, pattern formation, and transcription control (Woodgett, 2001; Yoo et al., 2006). Biological functions of AtSKs depend on the subgroup and include development, abiotic and biotic stress response, and hormone signaling (Jonak and Hirt, 2002). AtSK11 and AtSK12, in subgroup 1, have been implicated in regulating the early stages of floral meristem patterning (Dornelas et al., 2000). AtSK31, AtSK32 and other subgroup 3 AtSK orthologs in plants are expressed almost exclusively in flower organs and mature pollen grains (Tichtinsky et al., 1998; Tavares et al., 2002). AtSK32 has recently been shown to regulate cell elongation during flower development (Claisse et al., 2007). All subgroup 2 AtSKs (AtSK21, AtSK22 and AtSK23) have been found to be important regulators of brassinosteroid (BR) hormone signaling (see below). In addition, it has also been suggested that AtSK22 plays a role in activating salt stress response (Piao et al., 2001). The function of subgroup 4 AtSKs is still unknown.

The most intensively studied AtSKs belong to subgroup 2 and they have been shown to negatively regulate BR signaling. BRs are sterol-derived hormones found ubiquitously in the plant kingdom. They are involved in controlling cell division, elongation and differentiation, and regulating development (Clouse, 2002). The current model of the BR signaling pathway (Fig. 2.2) proposes that in the absence of the BR signal the subgroup 2 AtSKs are active (Ryu et al., 2007; Yan et al., 2009) and phosphorylate two closely related plant-specific transcription factors, BZR1 and BES1 (also known as BZR2) (Belkhadir and Chory, 2006). Inactivation of hyperphosphorylated BES1/BZR1 is achieved through several mechanisms including inhibiting their DNA-binding (Vert and Chory, 2006), promoting their export from the nucleus and cytoplasmic retention through binding of 14-3-3 proteins (Gampala et al., 2007; Ryu et al., 2007), and increasing their proteasome-mediated degradation (He et al., 2002; Yin et al., 2002).

BR signaling initiates when BR produced by neighboring cells binds directly to a leucine-rich repeat receptor-like kinase (LRR-RLK), BRI1 (Li and Chory, 1997; Kinoshita et al., 2005). The BR/BRI1 interaction dissociates BKI1, which is an inhibitor of BRI1, and allows BRI1 to autophosphorylate and trans-phosphorylate its co-receptor BAK1 (Wang and Chory, 2006). The active receptor-complex then phosphorylates BSK1 at the plasma membrane (Tang et al., 2008), which interacts with and activates BSU1 phosphatase (Kim et al., 2009). BSU1 dephosphorylates AtSK21, and possibly other AtSKs involved in BR signaling (see below), on a conserved tyrosine residue, leading to the AtSK's inactivation. This allows unphosphorylated BZR1 and BES1 to accumulate (Kim et al., 2009), bind to their target promoters and regulate gene expression. Since BZR1 is a repressor, its target genes, such as *CPD* and *DWF4*, are downregulated in the presence of BR (He et al., 2005); whereas, BES1 is an activator that induces the expression of its target genes, such as *SAUR-15* (Yin et al., 2005).

Recently it has been suggested that additional AtSKs are involved in BR signaling. Using an AtSK-specific inhibitor, De Rybel and collaborators (2009) suggested that subgroup 1 AtSKs and AtSK32 are probably involved in BR signaling, in addition to subgroup 2 AtSKs (De Rybel et al., 2009). Subgroup 1 AtSKs were also found to bind to BZR1 in yeast two-hybrid assays, and AtSK12 is capable of phosphorylating BZR1 *in vitro* and appears to be regulated by phosphorylation/dephosphorylation of the conserved tyrosine residue, further suggesting that subgroup I AtSKs are involved in BR signaling (Kim et al., 2009).

As C4 interacts with AtSK21 and AtSK23, and is phosphorylated *in vitro* by AtSK21 (Piroux et al., 2007), the possibility that C4 interferes with the function of AtSKs involved in BR signaling was explored herein. The BCTV C4 gene was also found to interact with an uncharacterized leucine-rich-repeat receptor-like kinase (LRR-RLK) in a yeast two-hybrid assay,

but the interaction could not be confirmed with *in vitro* binding assays, nor could direct phosphorylation of C4 by the LRR-RLK be shown (Piroux et al., 2007). The AC4 gene of *Tomato golden mosaic virus*, which does not contribute to the viruses' pathogenicity, also binds to AtSK21 and AtSK23, but not to the LRR-RLK, in yeast two-hybrid assays (Piroux et al., 2007), suggesting that the LRR-RLK/C4 interaction could be important for the pathogenicity of BCTV C4. The presence of an N-myristoylation motif on BCTV C4, which targets the protein to the plasma membrane (Piroux et al., 2007), further supports the possibility that the LRR-RLK/C4 interaction could occur *in vivo*. Other geminivirus proteins have been found to interact, and modify protein kinases; for example, the *Cabbage leaf curl virus* NSP inhibits the kinase activity of the receptor-like kinase NIK (Fontes et al., 2004), the NSP of several viral species interacts with the PERK-like receptor kinase NsAK to promote viral infection (Florentino et al., 2006) and with NIG, a GTPase that promotes nucleocytoplasmic shuttling of NSP (Carvalho and Lazarowitz, 2004), AL2/L2 genes interact with SNF1 kinase to inhibit its function (Hao et al., 2003).

The C4 genes of all monopartite geminiviruses studied to date play a role in pathogenicity (Stanley and Latham, 1992; Jupin et al., 1994; Krake et al., 1998; Gopal et al., 2007; Lai et al., 2009), whereas the AC4 genes of several bipartite geminiviruses have no effect on symptom development or viral replication (Sung and Coutts, 1995; Hoogstraten et al., 1996; Pooma and Petty, 1996; Bull et al., 2007). This difference could be due to the lack of the DNA-B component in monopartite geminiviruses, and therefore the absence of specialized NSP and MP protein encoded on it, driving the need for other viral proteins to substitute for them or evolve different functions to overcome the host's barriers. For example, mutations of the TYLCV C4 gene abolish systemic infection (Jupin et al., 1994) and it has been suggested that C4 and V2

function together as homologues of BC1 (MP) of bipartite geminiviruses (Rojas et al., 2001). A link between the C4 gene of *Beet severe curly top virus* (BSCTV), a close relative of BCTV, and cell cycle regulation was recently suggested, when ectopic expression of the BSCTV C4 gene lead to upregulation of RKP, a component of the 26S proteasome which is thought to be involved in regulating CKIs (Lai et al., 2009), which, as mentioned above, regulate the function of CDK/cyclin heterodimers. The AC4 gene of *African cassava mosaic virus - Cameroon* (ACMV-CM) has been implicated in altering *Arabidopsis* development through its binding to microRNAs (miRNAs) (Chellappan et al., 2005), which regulate many developmental processes by altering mRNA levels at different stages of development through sequence complementarity between the miRNA and the target mRNA (Bonnet et al., 2006). As mentioned previously, the C4 gene of BCTV appears to be responsible for inducing hyperplasia in host plants, since BCTV mutants that do not express C4 are no longer capable of inducing hyperplasia upon infection (Stanley and Latham, 1992) and ectopic expression of BCTV C4 leads to the production of enations in *N. benthamiana* (Latham et al., 1997). However, the full extent of the effects of C4 on plant development and the mechanism behind the induction of cell division have not been well studied to date and, therefore, is the focus of this dissertation.

Tables

Table 2-1. Geminivirus organization

Feature	<i>Begomovirus</i>	<i>Curtovirus</i>	<i>Mastrevirus</i>	<i>Topocuvirus</i>
Type member	Bean golden mosaic virus	Beat curly top virus	Maize streak virus	Tomato pseudo-curly top virus
Insect vector	Whitefly (<i>Bemisia tabaci</i> Genn.)	Leafhopper (<i>Circulifer tenellus</i> Baker)	Leafhopper (<i>Cicadulina mbila</i> Naude, and other <i>Cicadulina</i> sp.)	Treehopper (<i>Micrutalis malleifera</i> Fowler)
Host plants	Dicotyledoneous	Dicotyledoneous	Monocotyledonous, recently also dicotyledoneous	Dicotyledoneous
Genome	Predominantly bipartite, some monopartite	Monopartite	Monopartite	Monopartite
Genes encoded	<u>Monopartite:</u> Six 2 on V-sense 4 on C-sense <u>Bipartite:</u> Seven or eight DNA-A: 1 or 2 on V-sense 4 on C-sense DNA-B: 1 on V-sense 1 on C-sense	Seven 3 on V-sense 4 on C-sense	Four 2 on V-sense 2 on C-sense	Six 2 on the V-sense 4 on the C-sense
Special feature	80% of known geminiviruses belong to this group	Some species induce hyperplasia upon infection.	RepA Gene undergoes splicing. Does not encode REn.	

(Gutierrez, 1999; Fauquet and Stanley, 2003; Varma and Malathi, 2003)

Figures

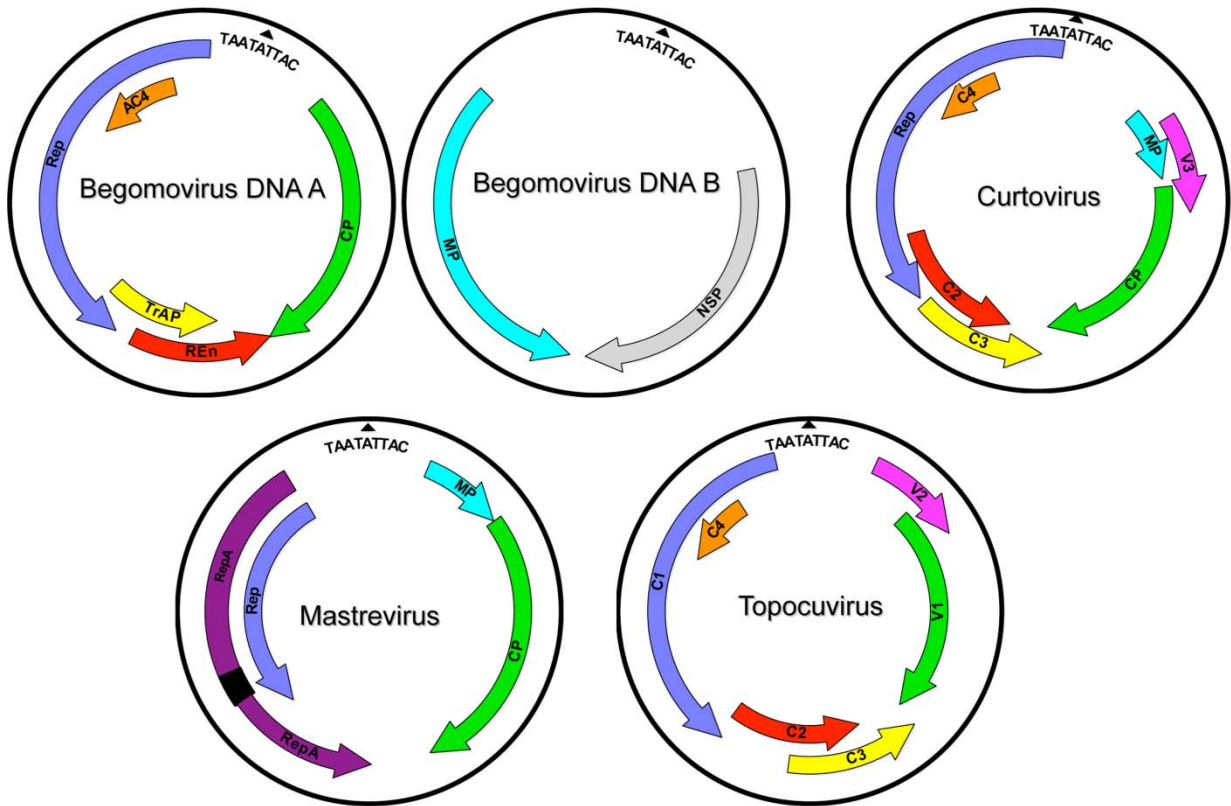


Fig. 2.1. Genomic organization of *Geminiviridae*. Geminiviruses have circular single stranded DNA genomes. Bipartite begomoviruses have two components (DNA A and DNA B), all other genera (Curtoviruses, Mastreviruses and Topocuviruses) and monopartite begomoviruses (not shown) have a single component. Genes encoded on the viral strand are designated with a V, those encoded on the complimentary strand are designated with a C. Genes with known function are designated as follows: CP, coat protein; MP, movement protein; NSP, nuclear shuttle protein; Rep, replication associated protein; REn, replication enhancer; TrAP, transcription activator. Nanonucleotide: TAATATTAC. See text and Table 2.1 for further information on geminivirus genera and gene function.

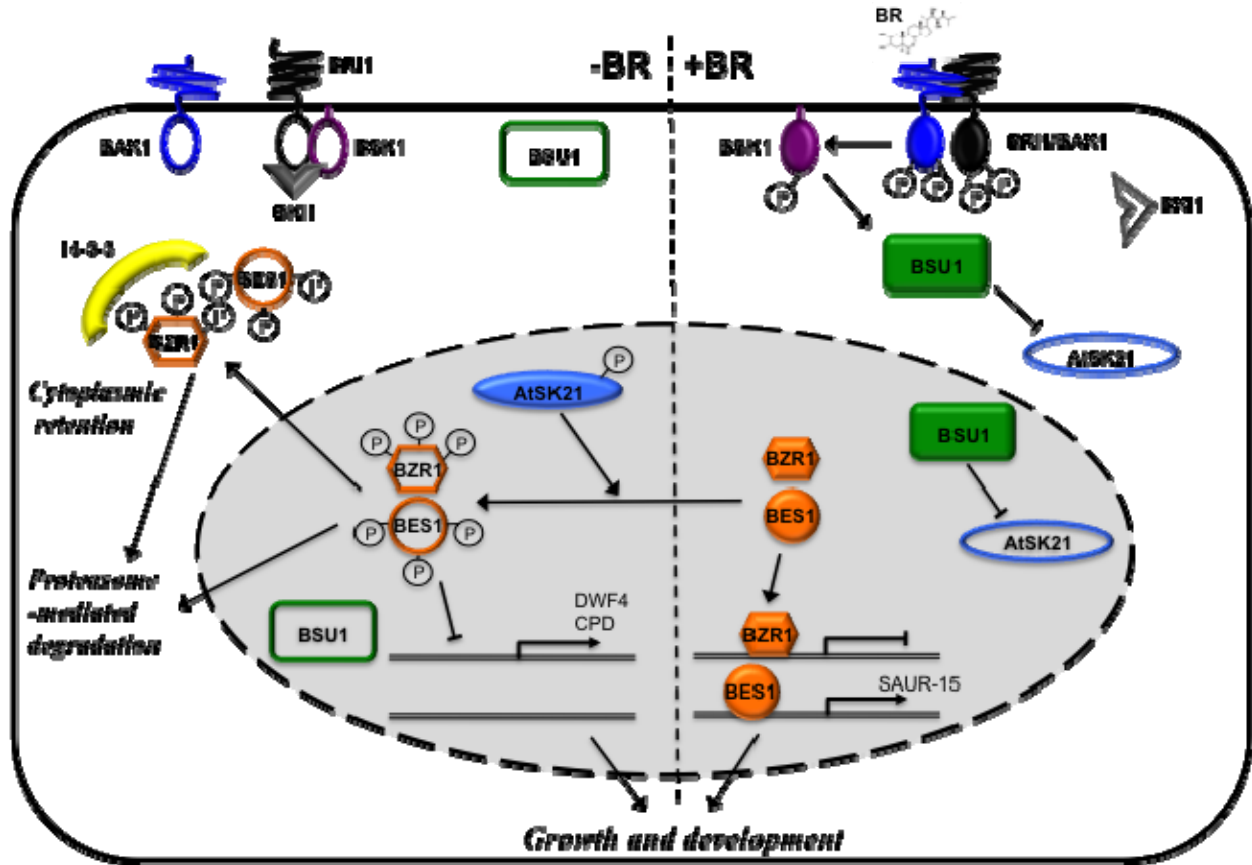


Fig. 2.2. Simplified model of the brassinosteroid (BR) signaling pathway. In the absence of BR (-BR), the BRI1 receptor is held in an inactive state by BKI1 inhibitor, allowing AtSK21 (and presumably other subgroup 2 and subgroup 1 AtSKs) to be active and phosphorylate BZR1 and BES1 transcription factors. Hyperphosphorylated BES1 and BZR1 are unable to bind to their target promoters, are excluded from the nucleus by 14-3-3 proteins, and are targeted for proteolytic degradation by the 26S proteasome. In the presence of BR (+BR), the BR hormone binds to the BRI1 receptor, causing a conformational change in BRI1, which dissociates BKI1 and allows BRI1 to associate with its co-receptor BAK1. Trans-phosphorylation by the BRI1/BAK1 receptor complex leads to its activation, and in turn, to the phosphorylation and activation of BSK1. Active BSK1 then activates BSU1 phosphatase, which inactivates AtSK21 (and other AtSKs, not shown here), allowing unphosphorylated BES1 and BZR1 to accumulate in the nucleus, and bind to their target promoters to regulate gene expression, ultimately leading to changes in growth and development. Filled objects indicate an active state, open objects an inactive state. Modified from (Kim et al., 2009).

CHAPTER 3

GEMINIVIRUS C4 PROTEIN ALTERS ARABIDOPSIS DEVELOPMENT¹

¹ **Mills-Lujan, K and C.M. Deom.** 2010. *Protoplasma* (DOI: 10.1007/s00709-009-0086-z).

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Abstract

The C4 protein of beet curly top virus (BCTV-B [US:Log:76]) induces hyperplasia in infected phloem tissue and tumorigenic growths in transgenic plants. The protein offers an excellent model for studying cell cycle control, cell differentiation and plant development. To investigate the role of the C4 protein in plant development, transgenic *Arabidopsis thaliana* plants were generated in which the C4 transgene was expressed under the control of an inducible promoter. A detailed analysis of the developmental changes that occur in cotyledons and hypocotyls of seedlings expressing the C4 transgene showed extensive cell division in all tissues types examined, radically altered tissue layer organization, and the absence of a clearly defined vascular system. Induced seedlings failed to develop true leaves, lateral roots, shoot and root apical meristems, as well as vascular tissue. Specialized epidermis structures, such as stomata and root hairs, were either absent or developmentally impaired in seedlings that expressed C4 protein. Exogenous application of brassinosteroid and abscisic acid weakly rescued the C4-induced phenotype, while induced seedlings were hypersensitive to gibberellic acid and kinetin. These results indicate that ectopic expression of the BCTV C4 protein in *A. thaliana* drastically alters plant development, possibly through the disruption of multiple hormonal pathways.

Introduction

Viruses in the family *Geminiviridae* have proven to be important not only as a major constraint to agriculture worldwide (Varma and Malathi, 2003; Seal et al., 2006), but also as a model for studying plant DNA replication and cell-cycle control (Hanley-Bowdoin et al., 1999; Gutierrez, 2002). Geminiviruses have small, mono- or bipartite, single-stranded DNA (ssDNA) genomes (each ~2.5 to 3.0 kb) with limited coding capacity (Fauquet et al., 2006) and require access to host-replication machinery to replicate their genomes.

In mature plants, the DNA replication machinery, undetectable in differentiated quiescent cells (Coello et al., 1992; Nakashima et al., 1998), is restricted to meristems (Martinez et al., 1992; Staiger and Doonan, 1993), or to endoreduplicating tissues. Geminiviruses are excluded from the apical meristems and do not have access to the replication machinery of these embryonic cells (Rushing et al., 1987; Nagar et al., 1995; Lucy et al., 1996; Peele et al., 2001). As a group, geminiviruses can infect a wide range of tissue types (Saunders et al. 2001; Wege et al. 2001), although the majority of geminiviruses are restricted to the vascular tissue (Horns and Jeske, 1991; Sanderfoot and Lazarowitz, 1996; Wang et al., 1996; Morra and Petty, 2000). Vascular-limited geminiviruses may induce synthesis of DNA replication machinery following infection of differentiated vascular tissue (Kong et al., 2000) or may access replication machinery in the vascular meristem. Geminiviruses that are not limited to vascular tissue infect and propagate in terminally differentiated leaf, stem, and root cells (Rushing et al., 1987; Nagar et al., 1995; Lucy et al., 1996; Morra and Petty, 2000). For geminiviruses to replicate in mature cells the viruses must reprogram the cell cycle so that host-replication machinery becomes available for virus replication.

In an approach reminiscent of small animal DNA tumor viruses (Felsani et al.), some geminiviruses encode factors that induce differentiated cells to enter the S-phase of the cell cycle where cellular DNA replication machinery is available for viral replication (Gutierrez, 2000; Hanley-Bowdoin et al., 2004). Geminiviruses encode proteins that bind to and modulate the function of the plant retinoblastoma-related protein (RBR) to generate an environment for viral replication. Geminivirus protein/RBR interactions have been demonstrated for the Rep and REN proteins of bipartite begomoviruses (Ach et al., 1997; Settlage et al., 2001; Arguello-Astorga et al., 2004) and the RepA protein of mastreviruses (Grafi et al., 1996; Xie et al., 1996; Horváth et al., 1998; Liu et al., 1999). RBR is involved in regulating differentiation, proliferation and endoreduplication in plants (Park et al., 2005). In quiescent cells, RBR negatively regulates cell cycle progression, in part, by binding to E2F-transcription factors and inactivating E2F-responsive genes. E2F-responsive genes control progression into S-phase of the cell cycle. Thus, geminivirus encoded proteins likely induce host DNA replication by interfering with the RBR/E2F transcriptional repression system, allowing cells to enter S-phase (Hanley-Bowdoin et al., 2004).

Evidence suggests that the cell cycle in infected plant cells is reprogrammed differently by different geminivirus genera. Some bipartite begomoviruses modify gene expression to allow for DNA replication primarily by endoreduplication (Bass et al., 2000; Hanley-Bowdoin et al., 2004). Mastreviruses induce DNA replication by endoreduplication, but also induce cell division in epidermal cells of young leaves (Desvoyes et al., 2006). In contrast, some monopartite geminiviruses induce extensive mitosis (Latham et al., 1997; Briddon and Markham, 2000; Hanley-Bowdoin et al., 2004), which gives the viruses access to cellular DNA replication machinery. Beet curly top virus [BCTV-B (US:Log:76)], a Curtovirus, referred to hereafter as

BCTV for brevity, is a phloem-limited geminivirus, which induces hyperplasia in the phloem tissue of infected spinach, sugar beet, *Nicotiana benthamiana* and *Arabidopsis* plants (Esau, 1976; Esau and Hoefert, 1978; Park et al., 2004). Mutations in the C4 gene of BCTV resulted in reduced disease symptoms on several host species (Stanley and Latham, 1992). In transgenic *N. benthamiana* the C4 transgene was capable of inducing a severe developmental phenotype that correlated with the relative amount of C4 transcripts and the sporadic development of tumorigenic growths on stems, petioles and leaves, and flowers (Latham et al., 1997).

C4 was shown to interact with two members of the *Arabidopsis* SHAGGY-related protein kinase family (AtSK), AtSK21 and AtSK23, and a leucine-rich repeat receptor-like kinase (LRR-RLK) of unknown function in yeast two-hybrid assays (Piroux et al., 2007).. The interaction between C4 and AtSK21 was confirmed through an *in vitro* binding assay (Piroux et al., 2007). The interactions between C4 and AtSK23 and the LRR-RLK proteins were not similarly confirmed since AtSK23 was unstable and full-length LRR-RLK was toxic when expressed in *E. coli*. AtSK21 phosphorylated C4 *in vitro* at unidentified threonine and serine residue(s), suggesting a regulatory role for phosphorylation in C4 function (Piroux et al., 2007).

AtSKs, which are encoded by a multigene family (ten members), are involved in diverse cellular processes including development, hormone signaling, and stress responses (Jonak and Hirt, 2002). Based on the sequence comparison of the catalytic domains the AtSK gene family was divided into four subgroups, of which subgroup 2 was composed of three members, AtSK21, AtSK22, and AtSK23 (Dornelas et al., 1998). Originally AtSK21 was shown to be a negative regulator of brassinosteroid (BR) signaling and, subsequently, AtSK22 and AtSK23 were shown to act redundantly with AtSK21 (Vert and Chory, 2006; Li and Jin, 2007; Ryu et al.,

2007). More recently evidence suggests additional AtSKs are also involved in regulating the BR signaling pathway (Rybel et al. 2009; Yan et al. 2009).

Brassinosteroids control many aspects of plant growth and development (Clouse and Sasse, 1998). Recent models suggest that AtSK21, and presumably AtSK22 and AtSK23, target two closely related transcription factors, BES1 and BZR1, which regulate expression of BR-target genes. In the absence of BR, AtSK21 hyperphosphorylates and inactivates BES1 and BZR1 by several mechanisms, including inhibition of their binding to DNA (Vert and Chory, 2006; Gampala et al. 2007; Gendron and Wang, 2007), increasing their rate of proteasome-mediated degradation (He et al, 2002; Yin et al, 2002), and promoting binding of 14-3-3 proteins that result in cytoplasmic retention (Bai et al. 2007; Gampala et al. 2007; Ryu et al. 2007). In the presence of BR, the hormone binds to the BR-receptor kinase (BRI1), which initiates a signaling cascade that results in the activation of BRI1 suppressor 1 (BSU1), a phosphatase (Kim et al, 2009). Activated BSU1 inactivates AtSK21 by dephosphorylating a conserved phosphotyrosine residue (amino acid residue 200) (Kim et al, 2009). The inhibition of AtSK21 allows the BZR1 and BES1 transcription factors to be dephosphorylated by BSU1, accumulate in the nucleus, and bind to BR-target gene promoters (Li and Deng, 2005; Belkhadir and Chory, 2006; Li and Jin, 2007).

The extent to which C4 induces cell division throughout various tissues *in planta* and to what extent C4 affects development has not been studied in detail. In this study we investigate the role that the BCTV C4 protein plays in abnormal plant development when expressed out of the context of a virus infection. A detailed phenotypic and structural analysis, at the organ and cellular levels, in *Arabidopsis* seedlings demonstrates that expression of the C4 protein leads to a severe development phenotype characterized by the loss of meristematic function, prolific cell

division in all organs analyzed, and the loss of cell-type differentiation. We show that multiple hormones alter the C4-induced phenotype, suggesting that C4 radically alters development in *Arabidopsis* by disrupting the plant hormonal network.

Materials and Methods

Plasmid Constructs

The BCTV-B (US:Log:76) C4 gene was amplified by polymerase chain reaction (PCR), using primers LOGAN45 and LOGANC43. PCR amplification of the C4 gene was performed in a 100 μ l reaction mix containing 2.5u/ μ l *Pfu*Ultra, 1X Pfu reaction buffer (Stratagene, La Jolla, CA), 0.2 μ M dNTP, and 0.8 μ M of each primer. PCR was programmed for 30 cycles of 60s at 95°C, 60s at 58°C, and 60s at 72°C, preceded by an initial denaturation for 4 min at 95°C, and followed by a 10 min extension at 72°C. The PCR product was digested with *Xho*I and *Spe*I and ligated into pER10 to give pERC4L. Genes cloned into pER10 (Zuo et al. 2000) are under regulatory control of an estradiol inducible promoter and the polyadenylation signal of the *rbcsS3A* gene. A non-translatable version of the C4 gene (C4nt) was generated by PCR using primers C4LNOATG (primer lacks the C4 start codon) and LOGANC43 and cloned into pER10 to give pERC4Lnt. C4 and C4nt genes were also cloned into pMON10098, following PCR-amplification using primers 35SLC45 and 35SLC43 and primers 98C4NT5 and 35SLC43, to give p98C4L and p98C4Lnt, respectively. Genes cloned into pMON10098 are under regulatory control of the constitutively expressed 35S promoter and the polyadenylation signal of the nopaline synthase gene. Plasmids pERC4L, pERC4Lnt, p98C4L, and p98C4Lnt were subsequently transformed into *Agrobacterium tumefaciens* strain ABI. The integrity of the C4

genes in all plasmids was confirmed by sequencing. PCR primers for cloning are indicated in Table S 3-1 (online, for published version only).

Generation of Transgenic *Arabidopsis* Plant Lines

Arabidopsis thaliana (*A. thaliana*) ecotype Sei-0 was used for all transformations (Clough and Bent, 1998). Harvested seeds were surface sterilized with 2 washes of 1 ml 100% ethanol, followed by 1 ml 50% bleach, 0.2% Tween-20, and three washes with sterile water. All steps were for 4 minutes. Seed was suspended in 1 ml of 0.1% sterile agarose, and spread on solid selection media [0.5X MS (Murashige and Skoog basal salt mixture), 0.8% tissue culture agar, 50 μ g ml⁻¹ Kanamycin]. Plates were placed at 4°C for 3 days, and then moved to a growth chamber (23°C, 16 hour light period). Transgenic plant lines were transplanted to soil and T₁ seeds were collected and germinated on selection media. Resistant seedlings from lines showing 3:1 (resistant:susceptible) segregation ratios were moved to soil for collection of T₂ seed. T₂ seeds were collected and screened on selective media for homozygous plants. Transgene copy number was confirmed by Southern blot analysis (Sambrook et al., 2001).

Induction of Transgenes

Seeds from homozygous lines were surface sterilized and germinated on solid induction media [1X MS, 3% sucrose, 0.8% agar, and varying concentrations of 17- β -estradiol (β -estradiol)]. A primary stock solution of 10mM β -estradiol was prepared in DMSO. Working solutions were prepared by diluting the primary stock solution in DMSO. Final concentrations of β -estradiol on induction media were 0.001, 0.01, 0.1, 1, 5, 10, and 0 μ M (0.001% DMSO only). Seedlings were monitored for up to 2 weeks. Additionally, seedlings were germinated in liquid

culture (Bae et al., 2005). Fifty sterile seeds were placed in a 250 ml Erlenmeyer flask with 50 ml liquid media (1X MS, 3% sucrose, 1X Gamborg's B5 Vitamins, 0.1 μ M MES) and grown for 7 days at 25°C with gentle shaking (100 rpm). For time course experiments, β -estradiol was added to a final concentration of 10 μ M after the initial 7-day germination period. Seedlings were harvested at 0, 1, 6, 12, 24, 48, 72 and 96 hours post-induction (hpi), washed with water, blotted dry, frozen in liquid nitrogen, and stored at -80°C. For experiments containing variable inducer concentrations, β -estradiol was added to a final concentration of 0.0, 0.001, 0.01, 0.1, 1, 5, and 10 μ M after the initial 7-day germination period. Seedlings were harvested at 24 hpi. Seedlings grown under liquid culture were under continuous light conditions. To determine the effect of BR and other plant hormones on the C4-induced phenotype solid induction media was amended with 1mM epibrassinolide (BL); 1 μ M indole-3-acetic acid (IAA, an auxin); 1 μ M kinetin; 1 μ M aminocyclopropane-carboxylic acid (ACPC, a precursor of ethylene); 1 μ M gibberellic acid (GA₃); or 0.5 μ M abscisic acid (ABA).

Microscopy Analysis

Seedlings germinated on solid media were viewed directly using a stereoscope fitted with a digital camera (Nikon DXM 1200C digital camera, Nikon SMZ 1500 stereoscope). Several pictures at different focus planes were taken of each seedling and digitally formatted using Auto-Montage Essentials (Synoptics, Ltd.) and Photoshop 6.0.

For histological observations of tissue sections, seedlings germinated in liquid media were fixed in 2.5% glutaraldehyde in buffer (50 μ M phosphate buffer pH 7.0) at 4°C, and then rinsed with buffer. A secondary fixation in 1% OsO₄ in buffer for 2 hours at 4°C was followed by rinsing in distilled water (dH₂O) and then overnight en-bloc staining with 0.5% aqueous

uranyl acetate. Tissue was then rinsed with dH₂O and dehydrated in a graded ethanol series (25 to 100%), followed by two incubations in 100% acetone. The tissue was infiltrated with Spurr's resin using serial gradient replacements (33%, 66% Spurr's in acetone, and 100% Spurr's), and finally polymerized by incubating for 48 hours at 60°C. Sections, of approximately 1 μm in thickness, were cut using an ultramicrotome (Ultracut E, Leica) equipped with a Diatome histoknife. Sections were mounted on Superfrost glass slides, and stained with toluidine blue. Pictures were taken using a Nikon DXM 1200C camera mounted on a Nikon Eclipse 50i compound microscope.

Vein visualization was performed (Zhou et al., 2007). Briefly, seedlings were sequentially treated in 95% (v/v) ethanol overnight, 7% (w/v) NaOH for 48h and 250% (w/v) chloral hydrate for 2h. Seedlings were observed with a stereoscope using dark-field illumination.

For scanning electron microscopy (SEM), primary and secondary fixations were performed as described above, followed by sequential dehydration (10 to 100% ethanol), and then dried using a Samdri critical point dryer (Tousimis, Rockville, MD). Samples were mounted onto aluminum stubs with carbon adhesive tabs, coated with gold, using the SPI-Model sputter coater, to a thickness of approximately 220Å, and viewed using a Zeiss ESEM 1450 EP (Zeiss, Germany) operating at 20 keV. All digitally captured images were formatted using Photoshop 6.0. Cell number and leaf area measurements were determined using ImageJ 10.2.

Western Analysis

Tissues were frozen in liquid nitrogen and powdered by grinding in a mortar and pestle. Powdered tissues were further ground in 2 volumes of ice-cold non-denaturing tricine buffer (200 μM Tris-HCl, pH 6.8, 40% glycerol). Ground tissue was transferred to a microfuge tube and

an equal volume of tricine sample buffer (200 μ M Tris-HCl, pH 6.8, 2% SDS, 40% glycerol, 2% β -mercaptoethanol, 0.04% coomassie brilliant blue G250) was added to each sample. The samples were heated in a boiling water bath for 5 min and centrifuged at 10,000 x g for 5 min. The supernatants were stored at -20 °C. Protein concentrations of extracts were determined using a CB-X Protein Assay kit (G-Biosciences, St. Louis, MO).

Proteins were separated in 16.5% Criterion Tris-Tricine/Peptide gels according to the manufacturer (Bio-Rad, Hercules, CA) and blotted onto nitrocellulose (Deom et al., 1987). The C4 protein was detected using affinity purified rabbit antiserum generated to the C4 peptide sequence SKEKFRSQISDYSTC (Sigma-Genosys, The Woodlands, TX) as the primary antibody. Anti-rabbit polyclonal antiserum, conjugated to alkaline phosphatase, was used as a second antibody for visualization (ProtoBlot II AP System, Promega, Madison, WI).

RNA Analysis (Northern blot and qRT-PCR)

Total RNA was extracted from 100 mg of tissue grown in liquid culture using an RNeasy Plant Mini kit (Qiagen, Valencia, CA). Electrophoresis, membrane transfer, and hybridization were performed using a Northern Max kit (Ambion, Austin, TX). Briefly, 6 μ g of RNA were mixed with formaldehyde load dye, denatured, loaded into wells of a 1% denaturing agarose gel, and separated. RNA was transferred onto a BrightStar Plus membrane (Ambion, Austin, TX), and crosslinked using a Stratalinker UV crosslinker (Stratagene, La Jolla, CA). A biotin (Bio-16-UTP, Ambion, Austin, TX) labeled RNA probe was generated using a MAXIscript kit (Ambion, Austin, TX) and T7 polymerase from template plasmid previously linearized with *Xho*I. Hybridization and high stringency washes were carried out at 68°C. Detection of the hybridized

probe was performed using the BioDetect BrightStar kit (Ambion, Austin, TX), followed by exposure to HyperfilmTM ECL X-ray film (Amersham Biosciences, Piscataway, NJ).

For quantitative real time PCR (qRT-PCR) analysis, total RNA was treated with DNaseI (New England Biolabs, Beverly, MA) following the manufacturer's instructions. cDNA was generated from 1.5 mg of DNase I treated total RNA using a Super Script II cDNA Synthesis kit (Invitrogen, Carlsbad, CA). The qRT-PCR reactions were performed in optical 96-well plates with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each 25.0 μ l reaction contained 12.5 μ l of 2X SYBR Green Master Mix reagent (Applied Biosystems, Foster City, CA), 5.0 μ l of a 1:50 dilution of cDNA, and 400 nM of each gene-specific primer. Primer pairs used for qRT-PCR are indicated in **Error! Reference source not found.** (online, for published version only). The following thermal profile was used for all PCR amplification reactions: 95°C for 10 min, 40 cycles of 15s at 95°C, and 60°C for 1 min. Melting curves were performed after cycle 40. ROXTM was used as a passive reference dye. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) and were normalized for the ACT2 gene. Experiments were carried out in biological triplicates.

Results

Expression of BCTV C4 Protein Leads to an Abnormal Developmental Phenotype in Arabidopsis Seedlings

Expression of the C4 protein in transgenic *N. benthamiana* induces a severe phenotype characterized by abnormal development and the appearance of tumors (Piroux et al., 2007). To better characterize the developmental defects induced by C4 *in planta* in a system more

amenable to genetic studies, we generated transgenic lines of *A. thaliana* expressing the C4 protein. As controls, we generated transgenic plants expressing a non-translatable C4 gene (C4nt) and an empty vector. The Sei-0 ecotype was used for this study since it was previously shown to be hypersensitive to BCTV with a symptom phenotype indicative of extensive hyperplasia (Lee et al., 1994). We initially attempted to express the C4 gene under the control of the constitutive 35S promoter, but were unsuccessful in recovering transgenic lines. Since constitutive expression of the wild-type C4 gene from the 35S promoter in Sei-0 was lethal, we generated transgenic Sei-0 plants expressing the C4 and C4nt genes under the control of a β -estradiol inducible-promoter (Zuo et al., 2000). To facilitate the study, transgenic homozygous lines with single copy insertions expressing the C4 gene (plant line IPC4-28) or the C4nt gene (plant line IPC4nt-12) under control of the inducible-promoter were selected for further study. In the absence of induction, growth and development of IPC4-28, IPC4nt-12, and wild-type *Arabidopsis* seedlings were indistinguishable (Fig. 3.1A). However, IPC4-28 seedlings showed severe stunting when germinated in the presence of 10 μ M β -estradiol and development was arrested at the cotyledon stage. In contrast, IPC4nt-12 and wild-type seedlings grown on induction media were indistinguishable from non-induced seedlings (Fig. 3.1A). Northern and western analyses indicated the presence of C4 transcripts in extracts from induced IPC4-28 and IPC4nt-12 seedlings (Fig. 3.1B, upper panel), but C4 protein was only detected in extracts from induced IPC4-28 seedlings (Fig. 3.1B, lower panel). These results demonstrate that the occurrence of severe development defects in induced transgenic *Arabidopsis* correlated with the presence of C4 protein.

The β -estradiol-inducible promoter has been shown to respond to a broad range of inducer concentrations and to respond rapidly following induction (Zuo et al., 2000). To

determine if the phenotypic response to C4 expression was all-or-nothing or dose dependent, we germinated seedlings in the presence of increasing concentrations of β -estradiol. IPC4-28 seedlings visualized under stereoscope microscopy at 2, 8 and 16 days postgermination (dpg) displayed an abnormal developmental phenotype that correlated with increasing concentrations of β -estradiol and increasing times postgermination (Fig. 3.2). At 2 dpg, a decrease in root hair formation was apparent at an inducer concentration as low as 0.01 μ M β -estradiol (Fig. 3.2E) and cotyledon leaves failed to separate in seedlings germinated on media containing inducer concentrations \geq 0.1 μ M β -estradiol (Fig. 3.2G, I, and K). By 8 dpg, cotyledon leaves on seedlings on media with inducer concentrations \geq 0.01 μ M had thickened and were developing calli (Fig. 3.2H, J, L). Therefore, in the presence of increasing concentrations of β -estradiol, seedlings showed a more severe phenotype, which included: stunting, a lack of shoot development, a lack of root growth, and eventually the development of extensive hyperplasia on the cotyledons and hypocotyls (Fig. 3.2P-R).

To confirm that the C4 transcript and protein were dose-dependent in transgenic seedlings, we germinated seedlings in liquid media and allowed them to grow for 7 days before adding β -estradiol at concentrations ranging from 0 μ M to 10 μ M β -estradiol. Seedlings were collected at 24 hours post-induction (hpi) and extracts were analyzed by northern and western analyses (Figure S 3.1A and B, respectively). C4 transcripts were not detected in extracts from non-induced IPC4-28 and IPC4nt-12, nor induced or non-induced control seedlings transformed only with vectors (line IPV-20 and CPV-25). However, transcripts were detected in IPC4-28 seedlings at an inducer concentration as low as 0.01 μ M β -estradiol and appeared to saturate at 5 μ M β -estradiol. High levels of C4nt transcripts were observed in extracts from induced IPC4nt-12 seedlings and in extracts from seedlings expressing C4nt from the constitutive 35S promoter

(CPC4nt-6). The response to β -estradiol concentrations observed in the northern analysis was reflected in a western analysis of extracts from induced IPC4-28 seedlings (Figure S 3.1B). No C4 protein was detected in extracts from induced control seedlings.

Likewise, we looked at how rapidly IPC4-28 seedlings responded to β -estradiol induction. Seven-day old IPC4-28 seedlings germinated and grown in liquid media were induced at 10 μ M β -estradiol, and then collected at times ranging from 0 to 96 hpi (**Error! Reference source not found.C**). C4 was not detected in extracts from non-induced IPC4-28 seedlings nor induced IPC4nt-12 control seedlings, but was detected as early as 6 hpi in extracts from induced IPC4-28 seedlings. The amount of C4 peaked at 24 hpi, and remained at a steady, or near steady, state through 96 hpi. The morphology of the seedlings changed with the length of induction, with observable differences beginning at 24 hpi (not shown), and becoming more severe by 48 hpi (Fig. 3.3A). The cotyledons and first pair of rosette leaves were epinastic and the primary taproot and lateral roots were shorter in induced IPC4-28 seedlings when compared to non-induced IPC4-28 control seedlings. The primary root was not only noticeably shorter, but also thicker towards the tip (Fig. 3.3A and D) than control roots (Fig. 3.3A and C). Lateral roots of induced seedlings were bent and twisted (Fig. 3.3M), and became noticeably thicker over time (Fig. 3.3G) compared to controls (Fig. 3.3J and F, respectively). Root hairs along the primary root were much shorter on induced seedlings (Fig. 3.3M) when compared to non-induced seedlings (Fig. 3.3J). The cotyledons, and especially the first pair of rosette leaves, curled downward [Fig. 3.3A (plant on right)], whereas the subsequent rosette leaves curled towards the adaxial side or corkscrewed (Fig. 3.3E), and the petioles were twisted and curled, in contrast to controls (Fig. 3.3B). In addition, the epidermis cells were different on both the adaxial and abaxial surface of the rosette leaves (Fig. 3.3H, I, K, L). The adaxial leaf surface of induced seedlings had larger

cells, resulting in a bumpy surface, especially at the base of trichomes (Fig. 3.3K), unlike non-induced leaves (Fig. 3.3H). On the contrary, the abaxial leaf surface of induced seedlings had clusters of small cells, and the overall shape of the epidermis cells was more rounded (Fig. 3.3L) than in non-induced seedlings (Fig. 3.3I). The morphology and development of non-induced IPC4-28 seedlings were identical to those of induced and non-induced control IPC4nt-12 and wild type seedlings (not shown). The change in morphology of differentiated cotyledons, rosette leaves, and roots after induction of C4 protein suggested that normal development was arrested and tissues were dedifferentiating. Similar results were obtained with seedlings germinated directly on induction media (see below).

C4 Differentially Stimulates Cell Division in Arabidopsis Cotyledons

To further investigate the thickening of cotyledons in IPC4-28 seedlings germinated on induction media, we examined cross sections of cotyledons from seedlings collected at 2, 3, and 8 dpg. Induced Sei-0, IPC4nt-12 and non-induced IPC4-28 seedlings were used as controls. At 2 dpg, the morphology of cotyledons from control and induced seedlings was similar (Fig. 3.4A, E, I and M). In cotyledons of the control seedlings, cell number in the mesophyll tissue did not appreciably change from 2-8 dpg, although normal cell expansion occurred (Fig. 3.4A-C, E-G, and I-K). However, in cotyledons of induced IPC4-28 seedlings a distinct phenotype was observed by 3 dpg (Fig. 3.4N). The mesophyll tissue was highly disorganized and was characterized by a large increase in cell number, a concomitant decrease in cell size, and the absence of intercellular spaces. By 8 dpg, the only discrete cell layer was in the middle of the cotyledon lamina, where a layer of smaller cells had formed (Fig. 3.4O). The increase in cell number occurred primarily in the mesophyll tissue of induced IPC4-28 seedlings (~19-fold; Fig.

3.4Q). The cells that were adaxial and abaxial to this central layer were composed of larger cells, some of which had expanded to a size similar to that of palisade and spongy mesophyll cells in cotyledons of control seedlings. The effects of the cotyledon tissue disorganization and increase in cell number were noticeable on the external surface of cotyledons of induced IPC4-28 seedlings (Fig. 3.4P), as seen with scanning electron microscopy (SEM), while the abaxial cotyledon surface of induced Sei-0, IPC4nt-12 and non-induced IPC4-28 control seedlings was smooth (Fig. 3.4D, H, L). The same phenotype was observed on the adaxial surface.

Expression of C4 Results in Loss of Meristem Function and Tissue Organization

The severe stunting observed in seedlings expressing C4 protein suggested that the shoot and root apical meristems (SAM and RAM, respectively) were not functioning normally. To investigate this further, we compared meristematic development in IPC4-28 seedlings germinated on induction media to non-induced IPC4-28 and induced IPC4nt-12 control seedlings. Normal SAMs, surrounded by newly developing leaves with trichomes on the adaxial surface, were seen in scanning electron micrographs of 5 day-old control seedlings (Fig. 3.5A and F). In contrast, the normal dome-shaped SAM was absent in C4-expressing seedlings and leaf development was aborted with only two severely stunted leaf primordia visible (Fig. 3.5K).

To determine how the expression of C4 was affecting the organization of the SAM tissue at the cellular level, we analyzed longitudinal sections from induced IPC4-28 and control seedlings at 2, 3, and 8 dpg under the light microscope. Normal development of SAM was observed in control seedlings (Fig. 3.5 B-D and G-I). At 2 and 3 dpg the morphology of the SAM in induced IPC4-28 seedlings was similar to that observed in control seedlings (Fig. 3.5B, G, L and Fig. 3.5C, H, M). However, by 8 dpg the dome shaped SAM, with adjacent developing

and expanding leaf primordia seen in control seedlings (Fig. 3.5D and I), was absent in induced IPC4-28 seedlings (Fig. 3.5N). In addition to affecting SAM development, C4 expression had a significant effect on tissue organization in IPC4-28 seedlings. At 3 dpv the tissue organization of the hypocotyl and cotyledons of induced IPC4-28 seedlings (Fig. 3.5M) was distinct from that observed in control seedlings (Fig. 3.5C and H), namely, it was characterized by an increase in cell number and a decrease in cell size. By 8 dpv, the phenotype was much more severe and the tissue organization was in disarray due to extensive cellular proliferation of most, if not all, cell types (Fig. 3.5N).

Expression of the C4 protein had a similar effect on the root apical meristem (RAM), as observed in longitudinal sections (Figure S 3.2). At 2 dpv, induced IPC4-28 seedlings still retained a somewhat normal RAM organization (Figure S 3.2G) when compared to control seedlings (Figure S 3.2A and D), but by 3 dpv tissue organization was lost at the root tip of induced IPC4-28 seedlings (Figure S 3.2H). Not only had the RAM become disorganized, but the differentiated tissue layers that are located distal to the RAM were also disorganized. The concentric tissue layer organization (i.e. epidermis, cortex, endodermis and vascular tissue) at the root tip was no longer distinguishable in induced IPC4-28 seedlings (Figure S 3.2H). At 8 dpv the structure of the root tip of the induced IPC4-28 seedling (Figure S 3.2I) had lost any resemblance to wild type root tip morphology (Figure S 3.2C and F). The cells in the region where the RAM would normally be were in complete disarray, cell files were no longer distinguishable, and the presence of many more cell layers in the cortex/vascular tissue region resulted in a thickening of the root tip. The progressive phenotypes observed in, and proximal to, the SAM and RAM in IPC4-28 seedlings following induction with β -estradiol are indicative of

prolific, uncontrolled cell division in tissue that is maturing and differentiating in control seedlings.

The procambium is the meristematic tissue that gives rise to the primary vascular tissue. To investigate if the procambium was also affected by C4 expression, we followed vascular tissue development in cross sections of cotyledon leaves. Vascular bundles could be distinguished at 2 dpv in cotyledons from induced IPC4-28 seedlings (Fig. 3.4), but were obscured at 3 dpv (Fig. 3.4N), and were completely undistinguishable at 8 dpv (Fig. 3.4), whereas vascular bundles were clearly visible at all stages of development in cotyledons of control seedlings (Fig. 3.4A-C, E-G, and I-K). To verify that vascular development was altered in C4-expressing seedlings, we examined the venation patterns in cleared cotyledons (Fig. 3.5). Cotyledons of control seedlings displayed a typical wild-type vein pattern at 8 dpv (Fig. 3.5E and J), while veins were absent in cotyledons of induced IPC4-28 seedlings (Fig. 3.5O).

Taken together, the results from the longitudinal sections of the SAM and RAM, the cross sections from the cotyledons, and the vein visualization assays indicate that normal meristematic development is lost in IPC4-28 seedlings expressing C4 protein. Furthermore, tissue and cellular organization in differentiated and maturing tissues are lost in induced IPC4-28 seedlings and extensive cell division is occurring in these regions.

Expression of C4 Protein Leads to Loss of Cell Identity and Differentiation

Further characterization, using SEM analysis, of seedlings germinated directly on media with and without inducer revealed that the cotyledons of induced IPC4-28 seedlings at 5 dpv had bumps on the epidermis surface. These bumps were composed of rounded epidermal cells (Fig. 3.6E) that did not show the interlocking, jigsaw-puzzle pattern with neighboring cells typical of

wild-type pavement cells, as seen in control seedlings at 5 dpv (Fig. 3.6A and C). At 20 dpv, cells were observed that protruded out from the epidermis surface plane and formed finger-like structures (Fig. 3.6G). There were no stomatal complexes visible on the abaxial (Fig. 3.6E and G) or adaxial surface (not shown) of cotyledons from induced IPC4-28 seedlings, in contrast to controls (Fig. 3.6A and C). The overall size of individual pavement cells on cotyledons of induced IPC4-28 seedlings appeared similar to those on control seedlings (Fig. 3.6C and E), however, the swollen and bumpy nature of the abaxial surface of induced IPC4-28 cotyledons was indicative of an increased surface area and, as indicated in Fig. 3.4Q, a corresponding increase in cell number. Interestingly, cell size was increased on the adaxial epidermis of rosette leaves in IPC4-28 seedlings germinated without induction and observed at 48 hpi (Fig. 3.3K).

In addition to the lack of stomata, induced IPC4-28 seedlings produced less root hairs in the hypocotyl/root transition zone (Fig. 3.6F and H) when compared to control seedlings (Fig. 3.6B and D). Also, root hairs normally seen along the entire length of the root were absent from IPC4-28 seedlings germinated in the presence of inducer. Similar aberrant root hair development was noticed in IPC4-28 seedlings that were germinated without inducer and then observed at 48 hpi (Fig. 3.3M). The absence of normal stomata and root hairs in IPC4-28 seedlings suggests that the C4 protein has altered the normal development pathway for these two structures.

Lateral roots developed normally in control seedlings at 5 dpv (Fig. 3.6B and D), but were not observed in induced IPC4-28 seedlings (Fig. 3.6F and H). As mentioned above, IPC4-28 seedlings germinated in liquid media, induced after 7 days, and collected between 24 and 48 hpi had shorter and less abundant lateral roots (Fig. 3.3). Lateral root initiation requires that founder cells be specified within the pericycle and that these founder cells divide in specific patterns to give rise to a new lateral root (De Smet et al. 2006). The loss of cell layer

organization at the RAM (Figure S 3.2), and the lack of, or abnormal lateral root initiation, suggests that both meristem maintenance and initiation are lost in roots of induced IPC4-28 seedlings.

Effects of Exogenously Applied Hormones on the C4-Induced Phenotype

The C4 protein was shown to interact with AtSK21 in yeast two-hybrid and *in vitro* binding assays, suggesting a role for C4 in regulating the BR signaling pathway (Piroux et al., 2007). To investigate a possible *in planta* role for C4 protein in the BR signaling pathway we germinated IPC4-28 seedlings on induction media amended with brassinolide (BL), the most active endogenous brassinosteroid (Fig. 3.7), and compared their development to that of control IPC4nt-12 seedlings germinated under the same conditions. At 5 dpg, control IPC4nt-12 and IPC4-28 seedlings germinated in the presence of BL had stunted roots and formed a hook at the base of the hypocotyl (Fig. 3.7B and E). By 15 dpg, control IPC4nt-12 seedlings germinated in the presence of BL only or BL and β -estradiol had shorter roots that were twisted and the cotyledons were epinastic (Fig. 3.7H and I). The same phenotype was seen for IPC4-28 seedlings germinated with BL only (Fig. 3.7K). The addition of β -estradiol had no effect on IPC4nt-12 seedling development (Fig. 3.7A and G), which was identical to IPC4nt-12 and IPC4-28 seedling development in the absence of β -estradiol at similar dpi (compare Fig. 3.7A and G with Fig. 3.1, Fig. 3.2M-N, and Figure S 3.1A-D), nor did it alter the effects of BL treatment (Fig. 3.7C and I). However, exogenous BL had an effect on the development of induced IPC4-28 seedlings. Most notably the cotyledons of induced IPC4-28 seedlings, normally closed at 5 dpg (Fig. 3.2L and Fig. 3.7D), were open in seedlings germinated on BL-amended induction media (Fig. 3.7F). Induced IPC4-28 seedlings showed extensive and increasing hyperplasia throughout the

cotyledons and hypocotyl (Fig. 3.2P, Fig. 3.7J and 7M), which was greatly reduced in the presence of BL (Fig. 3.7L and N). Furthermore, by 15 dpv IPC4-28 seedlings germinated on BL-amended induction media gave rise to what appeared to be leaf primordia at the position where the SAM would normally be located (Fig. 3.7L). These leaf primordia continued to increase in size and developed structures similar to trichomes by 20 dpv (Fig. 3.7N and P), suggesting that not all tissue differentiation was lost. Together these results suggest that the addition of exogenous BL is able to weakly rescue the C4-induced phenotype, by decreasing hyperplasia, and allowing some tissue differentiation.

AtSK21 negatively regulates two transcription factors that in turn regulate the expression of, at least some, BR-responsive target genes such as *CPD*, *DWF4*, and *SAUR-15* (Wang and Chory, 2006). To provide further *in planta* evidence that the C4 protein modifies the BR signaling pathway, the expression of *CPD*, *DWF4*, and *SAUR-15* was compared in induced and non-induced IPC4-28 seedlings over time by quantitative real-time PCR (qRT-PCR) analysis (Fig. 3.8). Induction of the C4 gene was rapid and strong (Fig. 3.8A). C4 transcripts increased by ~187 fold at 3 hpi, and reached near maximum levels (~1,042 fold) by 24 hpi. The expression of *SAUR-15* also increased in induced IPC4-28 seedlings over time, with approximately a 3-fold increase in expression at 3 hpi and a maximum increase of ~13 fold at 24 hpi (Fig. 3.8B). The expression of both *CPD* and *DWF4* decreased with respect to the control following C4 induction, with maximum repression of ~10 fold in *CPD* and ~9 fold in *DWF4* expression levels by 24 hpi (Fig. 3.8C and D). These results indicate that C4 expression alters regulation of the BR signaling pathway.

Because BL had an effect on the C4 phenotype, we also examined the effect C4 had on the expression of *SAUR-15*, *CPD*, and *DWF4* in the presence of BL compared to BL alone. In

the presence of BL alone, *SAUR-15* transcripts increased ~7 fold with respect to the control at 6 hpi and then slowly decreased (Fig. 3.8B). The expression of *SAUR-15* transcripts was enhanced from ~13 fold in the presence of β -estradiol alone to ~22 fold in the presence of both β -estradiol and BL by 24 hours. By comparison, a much more rapid initial repression of *CPD* and *DWF4* expression, with respect to C4, was observed in the presence of BL (Fig. 3.8C and D). Maximum repression of *CPD* (~35 fold) and *DWF4* (~10 fold) were observed at 3 hours after addition of BL. Similar to BL treatment only, a rapid repression in expression levels of *CPD* (~45 fold) and *DWF4* (~20 fold) were observed at 3 hours following addition of β -estradiol and BL, but unlike BL alone, the expression levels of *CPD* and *DWF4* continued to decrease through 48 hours (~80 and ~60 fold, respectively). Therefore, repression of *CPD* and *DWF4* was enhanced when C4 and BL were both present compared to when C4 or BL were present alone.

Integration of the BR and auxin pathways and the BR and ABA pathways has been reported. AtSK21 was shown to inactivate Auxin Response Factor 2 (ARF2), a repressor of auxin-induced gene expression, by phosphorylation (Vert et al. 2008) and a large proportion of BR-responsive genes were shown to be regulated by ABA, likely through AtSK21 (Zhang et al. 2009). In addition, evidence suggests that crosstalk occurs between the BR and ethylene pathways (Gendron et al, 2008). Therefore, we looked at C4-induced and non-induced seedlings in the presence of IAA (an auxin), ABA, or ACPC (a precursor of ethylene) to see if exogenous addition of the hormones would have an effect on the C4-induced phenotype (Figure S 3.4). The addition of IAA did not rescue the C4-induced phenotype. To the contrary, IPC4-28 seedlings germinated on induction media amended with 1 μ M IAA had shorter, more hyperplastic roots (Figure S 3.4R and T) than IPC4-28 seedlings germinated on induction media only (Figure S 3.4F and H). In the presence of 0.5 μ M ABA, induced IPC4-28 seedlings were initially similar to

induced IPC4-28 seedlings in the absence of ABA. However, at 15 dpg, cotyledons on induced IPC4-28 seedlings amended with ABA were less hyperplastic (Figure S 3.4L) relative to those on IPC4-28 seedlings grown only on induction media (Figure S 3.4H). ACPC had no effect on control or IPC4-28 seedlings in the absence or presence of inducer (data not shown).

To determine if other plant hormones could affect the C4-induced phenotype, we germinated IPC4nt-12 and IPC4-28 seedlings on solid media containing GA₃ and kinetin in the absence or presence of inducer. Induced IPC4-28 seedlings were hypersensitive to GA₃ and kinetin. While GA₃ had no effect on control seedlings in the presence or absence of inducer, induced IPC4-28 seedlings in the presence of GA₃ were phenotypically similar to induced IPC4-28 seedlings until approximately 4 dpi then turned white and were dead by 6 dpi (Figure S 3.4N and P). In the presence of kinetin all control seedlings were stunted and exhibited extensive anthocyanin production relative to control seedlings not amended with kinetin. Induced IPC4-28 seedlings in the presence of kinetin were stunted, but had a hyperplastic phenotype similar to induced IPC4-28 seedlings until approximately 6-7 dpi then began to turn white and were dead by approximately 8-9 dpi (data not shown).

Discussion

Role of the C4 Protein in Aberrant Arabidopsis Development

The protein encoded by the C4 gene of BCTV induces cell division, which results in enations and a severe phenotype, when expressed in transgenic *N. benthamiana* plants (Latham et al., 1997). However, the extent to which C4 induces cell division throughout various tissues and the extent to which C4 affects development of different tissues *in planta* have not been

studied in detail. To better understand the influence of C4 on plant development, we performed a detailed structural analysis of various tissues in *Arabidopsis* seedlings expressing the C4 protein under control of an inducible promoter. Our results indicate that expression of C4 led to aberrant development in all tissue types examined from induced IPC4-28 seedlings, which resulted in a severe phenotype. First, entire tissue layers were disorganized. Cell division was not restricted to the meristems, with extensive cell division occurring throughout the cotyledon leaf blade, the hypocotyl, and the root tip, including the cell elongation and maturation zones. Second, seedlings germinated on induction media lost meristem function. Both the SAM and RAM were severely stunted, with aerial organ development and primary root and lateral root elongation lacking in each meristem, respectively. The meristematic activity of the procambium was also lost, since cotyledons did not develop vascular tissues. Third, lateral root development was not observed in IPC4-28 seedlings germinated directly on induction media or was reduced and stunted in seedlings that were induced after initial germination on liquid media without hormone. Lateral root formation requires the specification of founder cells within the pericycle and the division of these cells in characteristic patterns (De Smet et al., 2006). It has been suggested that lateral root initiation requires signals from the RAM (De Smet et al. 2006). Since RAM organization and development was abnormal in induced IPC4-28 seedlings, the RAM signal necessary for lateral root initiation likely was absent. Finally, the development of specialized structures, such as stomata and root hairs, was abnormal. Stomata are produced from a dedicated cell lineage during the development of shoot epidermis (Bergmann and Sack 2007), while the patterning of root hairs requires a position-dependent signaling mechanism in which epidermal cell fate is determined by the position of epidermis cells relative to the underlying cortical cells (Serna, 2005). In IPC4-28 seedlings germinated on induction media, the cotyledon epidermis

organization was atypical and the root epidermis and underlying cortex were in disarray and no longer organized into neat cell files, indicating the signals required for the normal development of both stomata and root hairs were absent or disrupted.

Hormone Pathway Interruptions

One mechanism by which the C4 protein has been proposed to function is through an interaction with the BR signaling pathway. C4 was shown to interact with AtSK21 and AtSK23 in yeast-two hybrid assays and with AtSK21 in an *in vitro* binding assay (Piroux et al., 2007). Subgroup 2 AtSKs are functionally redundant and negatively regulate BES1 and BZR1 transcription factors (Vert and Chory, 2006; Li and Jin, 2007; Ryu et al., 2007), which in turn regulate the expression of BR-responsive target genes such as *CPD*, *DWF4*, and *SAUR-15* (Wang and Chory, 2006). Even though a direct interaction between the subgroup 2 AtSKs and C4 has not been shown *in vivo*, our results strongly support a role for the C4 protein in modulating components of the BR signaling pathway, presumably the AtSKs, *in planta*. Our results show that upon C4 induction the expression of *SAUR-15* is upregulated and both *CPD* and *DWF4* are downregulated. This is consistent with C4 regulating AtSK21 phosphorylation of BES1 and BZR1. As might be expected, when BL and C4 were present together there was an enhanced effect on the expression levels of *SAUR-15*, *CPD* and *DWF4*.

Presently we do not have an explanation as to why the addition of exogenous BL results in a weak recovery phenotype in C4-induced seedlings. Indeed, it seems counter intuitive that the addition of BL would have an effect on rescuing the C4-induced phenotype, since, like BL, C4 appears to be a negative regulator of AtSK. While AtSK21 is found in many cellular compartments, it is the nuclear fraction that has the greatest effect on BR signaling (Belkadir

and Chory 2006). In contrast, C4 is localized to the plasma membrane and, at least *in vitro*, has been shown to bind to and be phosphorylated by AtSK21. Therefore, it is likely that C4 and BL regulate AtSK21 by different mechanisms, which may have varying effects on modulating AtSK21 function(s). Indeed, the negative regulatory effect that BL has on AtSK21 may compete against C4 to attenuate the C4 phenotype.

Individual knockout mutants of the subgroup 2 AtSK proteins, AtSK21, AtSK22 and AtSK23, show no visible phenotypes; however, a triple knockout mutant for subgroup 2 AtSK proteins was phenotypically characterized by long, bending leaf petioles and curling leaves (Vert and Chory, 2006). The requirement of a triple knockout mutant for a phenotype suggested that the subgroup 2 AtSK proteins are functionally redundant (Vert and Chory, 2006) and, by inference, that C4 might interact with all three subgroup 2 AtSK proteins. However, the C4-induced phenotype in transgenic *Arabidopsis* is very different than the non-hyperplastic phenotype of the subgroup 2 triple knockout mutant. Therefore, it does not appear that the severe development phenotype induced by C4 is solely due to the C4 protein disrupting the BR-signalling pathway, but is likely due to C4 modulating the function of an additional developmental pathway(s), which results in a pleiotropic phenotype. However, an intriguing possibility is that C4 interacts with multiple members of the AtSK family, contributing to or resulting in the C4-induced phenotype. In the future it will be interesting to determine how many members of the AtSK family the C4 protein interacts with and if interaction with other non-subgroup 2 AtSK members correlates with the hyperplasia induced by C4.

Recent advances have been made in understanding how crosstalk occurs between hormones with the promise of integrating hormone signaling pathways into regulatory networks (Santner and Estelle 2009). While it is well known that AtSK21 is involved in regulating the BR

signaling pathway, it is becoming increasingly clear that AtSK21 is a regulatory element shared by multiple hormone pathways (Nakamura et al. 2003; Hardtke 2007; Vert et al. 2008; Zhang et al. 2009). Crosstalk between the BR and auxin pathways was shown to occur through the direct action of AtSK21 on ARF2, a member of the Auxin Response factor family of transcription regulators (Vert et al., 2008). More recently, it was shown that ABA regulates the BR signaling pathway, likely through AtSK21, via ABI1 and ABI2, members of the PP2C family of serine/threonine phosphatases (Zhang et al. 2009). However, preliminary experiments (Figure S 3.4) suggested that the addition of IAA or ABA in the presence of C4 weakly affected the C4-induced phenotype. IAA increased hyperplasia in roots of seedlings expressing C4, while ABA induced a weak recovery phenotype in cotyledons. Interestingly, induced IPC4-28 seedlings were hypersensitive to GA₃ and kinetin. The hypersensitivity to kinetin might be a secondary effect since kinetin also had a significant effect on the development of control seedlings in the absence of induced C4. However, GA₃ had no visible effect on control seedlings in the absence of inducer, suggesting that the phenotype caused by GA₃ on C4-expressing seedlings may dependent on a C4-mediated function. Taken together, our results suggest that C4 has an effect on a number of hormone pathways, which may or may not be limited to crosstalk points in the context of hormonal regulatory networks.

Another cellular protein that C4 is likely to interact with *in planta* is the LRR-RLK-like protein that was shown to interact with C4 in a yeast two-hybrid screen (Piroux et al., 2007). An LRR-RLK/C4 interaction could have biological significance *in planta* and contribute to the pleiotropic phenotype induced by C4. Although the function of the LRR-RLK protein is unknown, LRR-RLK family members typically play important roles in many plant signal-transduction pathways (Tichtinsky et al., 2003). Therefore, it is conceivable that an LRR-

RLK/C4 interaction could disrupt the regulation of a developmental process independent of BR signaling. Interestingly, the nuclear shuttle proteins (NSP) of three bipartite begomoviruses were shown to interact with members of the NSP-interacting kinase (NIK) family (NIK1, NIK2, NIK3) of LRR-RLK proteins from tomato and soybean (Fontes et al., 2004; Mariano et al., 2004). NIK1 and NIK3 knockout lines of *Arabidopsis* were more susceptible to an attenuated Cabbage leaf curl virus (CaLCuV), suggesting that NSP might be involved in suppressing NIK-mediated antiviral activity (Fontes et al., 2004). Therefore, it is conceivable that C4 binds to an LRR-RLK protein, such as that described by Piroux et al (2007), and inhibits its kinase activity. Alternatively, the LRR-RLK protein could regulate the function of C4 through phosphorylation, as has been suggested for the C4/AtSK21 interaction (Piroux et al., 2007).

The AC4 proteins of ACMV and EACMCV, two closely related begomoviruses, were identified as suppressors of post-transcriptional gene silencing (PTGS; (Chellappan et al., 2005) and systemic PTGS (Fondong et al., 2007), respectively. AC4 of ACMV, but not EACMCV, acts as a suppressor of PTGS by binding to, and inactivating, miRNA159, (Vanitharani et al., 2004; Chellappan et al., 2005), which regulates the expression of the functionally redundant transcription factors MYB33 and MYB65 (Millar and Gubler, 2005; Palatnik et al., 2007). The expression of ACMV AC4 protein resulted in reduced levels of miR159, increased levels of *MYB33* transcripts and a severe developmental phenotype in transgenic *Arabidopsis* plants (Chellappan et al., 2005). Unlike AC4 of ACMV, C4 does not appear to target miR159 for destruction, since the levels of *MYB33* and *MYB65* transcripts do not change in transgenic *Arabidopsis* seedlings that express C4 protein (Fig. 3.8A). However, we cannot rule out the possibility that C4 interacts with other miRNAs or that it could be a suppressor of PTGS through

an entirely different mechanism, as is the silencing mechanism utilized by AC4 of EACMCV (Chellappan et al., 2005).

Role of C4 in Induction of Mitosis

IPC4-28 seedlings germinated on induction media showed an increased cell number in cotyledons, hypocotyls and root tips when compared to non-induced IPC4-28 seedlings. C4-induced cell division was verified by the expression patterns of genes encoding B1- and B2- type cyclins, CYCA1;1 and CYCB1;4, respectively, and a B2-type cyclin-dependent kinase, CDKB2;2, that are upregulated in the G₂/M transition and the M phases of the cell cycle (Menges et al., 2005). Significant changes (≥ 2 -fold) in expression of the three mitotic genes were detected by 24 hpi of C4 (Figure S 3.3). We chose mitotic markers to be able to distinguish between induction of mitosis and endoreduplication. Microarray analysis of *Arabidopsis* gene expression in response to infection by CaLCuV has identified >5000 genes that are differentially expressed at 12 days post inoculation (Ascencio-Ibanez et al., 2008). Similar to RNA viruses, DNA viruses induce genes involved in general pathogen responses. However, CaLCuV infection also altered expression of a number of cell cycle genes, preferentially inhibiting genes typically expressed during the G1 and M phases of the cell cycle and activating those expressed during S and G2 phases. Supporting evidence indicated that CaLCuV infection induced the endocycle and that over-expression of genes that promote mitosis inhibited CaLCuV infection. Interestingly, the C4 gene is not necessary for infection, at least in some hosts (Latham et al., 1997), suggesting that the endocycle, and not mitosis, is required for replication of geminiviruses that induce cell division (Ascencio-Ibanez et al., 2008). Presently, the purpose of the C4 protein in virus

infection is unknown, although it may be required for virus replication in natural hosts, or at least increase the efficiency of replication.

In contrast to animals, disturbances in mitotic control of plants are rarely associated with hyperplasia even though plant cells have the capacity to dedifferentiate to totipotency, proliferate, and re-differentiate. In this study we provide a detailed phenotypic and structural analysis of the extreme developmental changes induced by expression of the BCTV C4 protein and provide *in planta* evidence supporting a role for the C4 protein in interfering with multiple hormone pathways to induce the developmental changes. We are in the process of identifying the specific host protein(s) that the C4 protein interacts with *in planta* and to better define the mechanism by which C4 induces such a severe phenotype. While little is known about the regulatory roles of the C4 protein, this protein provides an important tool to gain insights and knowledge about host factors and pathways involved in cell cycle regulation and to elucidate the relationship between the plant cell cycle and development.

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Conflict of Interest The authors declare that they have no conflict of interest.

Tables

Table S 3-1 Primer pairs used for cloning and qRT-PCR analysis.

PCR or qRT-PCR target gene	Primer		Cloning restriction site ^a	qRT-PCR product size (bp)
	Name	Sequence		
BCTV-C4	LOGAN45	CCGCTCGAGAACAATGGGCAACCTCA TCTCCACGTCC	<i>XhoI</i>	
BCTV-C4	LOGANC43	GGACTAGTTTAACGCCTTGGCATATG ATCG	<i>SpeI</i>	
BCTV-C4	C4LNOATG	CGGCTCGAGAACAGGCAACCTCATCT CCACGTCCTGC	<i>XhoI</i>	
BCTV-C4	35SLC45	GCTCTAGAAACAATGGGCAACCTC AT CTCCACGTCC	<i>XbaI</i>	
BCTV-C4	35SLC43	CGGGATCCTTAACGCCTTGGCATATG AGTCG	<i>BamHI</i>	
BCTV-C4	98C4NT5	CGGGAGCTCTGGGCAACCTCATCTCC ACGTCCTGC	<i>SacI</i>	
BCTV-C4	C4qRTPCR F	AAATCCAGCTCCGACGTCAAGT		130
	C4qRTPCR R	ACGCCTTGGCATATGAGTCGTT		
CDKB2;2 (At1g20930)	CDKB2-2 F	ACCAAACGAAGAAGTTTGGCCTGG		88
	CDKB2-2 R	AGCTGTGGAGAGACTCAACGGTTT		
CYCA1;1 (At1g44110)	CYCA1-1 F	TACTAGTAGCTGCTTCCGCCATT		77
	CYCA1-1 R	AACGTCGAGTTCCATGGTCGTCTT		
CYCB1;4 (At2g26760)	CYCB1-4 F	ACCTTAAGAGCTCGAAGCAAGGCT		101
	CYCB1-4 R	ACGTATTCCACAGCAGCCAGTTCA		
CPD (At5g05690)	CPD FOR	TAATGAGACGCTACGAGTGGCTAAC		133
	CPD REV	GTCTAAATGAACCGCTCTAAACGAT		
DWF4 (At3g50660)	DWF4 For4	AAACAACGGAGCGTCATCCTCA		102
	DWF4 Rev4	AGCTCTGAACCAGCACATAGCCTT		
SAUR-15 (At4g38850)	SAUR For3	TCATGGCGGTCTATGTAGGAGAGA		146
	SAUR Rev3	TGTTAAGCCGCCATTGGATGA		
MYB33 (At5g06100)	MYB33 For	ATGAAACTCCACCAAGGCAGAGA		89
	MYB33 Rev	AAGCCAGCTCGATGCAAGCAAAA		
MYB65 (At3g11440)	MYB65 For	AATGTACCACGTGGCAGCGAAA		112
	MYB65 Rev	AACCACCTGCACTAAGTTGCGA		

^aRestriction site underlined in the primer sequence.

Figures

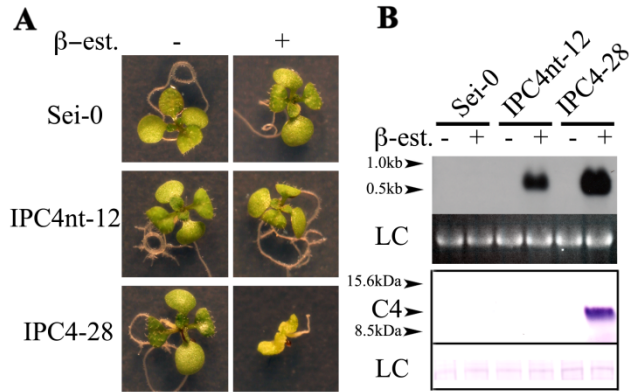


Fig. 3.1 Inducible expression of the C4 protein leads to an abnormal developmental phenotype in transgenic *Arabidopsis*. **(A)** Non-transformed ecotype Sei-0, transgenic control line IPC4nt-12 and IPC4-28 seedlings germinated on solid media with (+) and without (-) 10 μ M β -estradiol. **(B)** Northern blot analysis on total RNA extracted from seedlings in **(A)** and rRNA loading control (upper panel). Molecular size markers are indicated. Western blot analysis on total protein extracted from seedlings in **(A)** and non-specific protein band loading control (lower panel). Images and RNA were taken from seedlings at 12 dpv and the experiment was repeated 3 times. Protein molecular mass markers and C4 are indicated. β -est., β -estradiol; LC, loading control

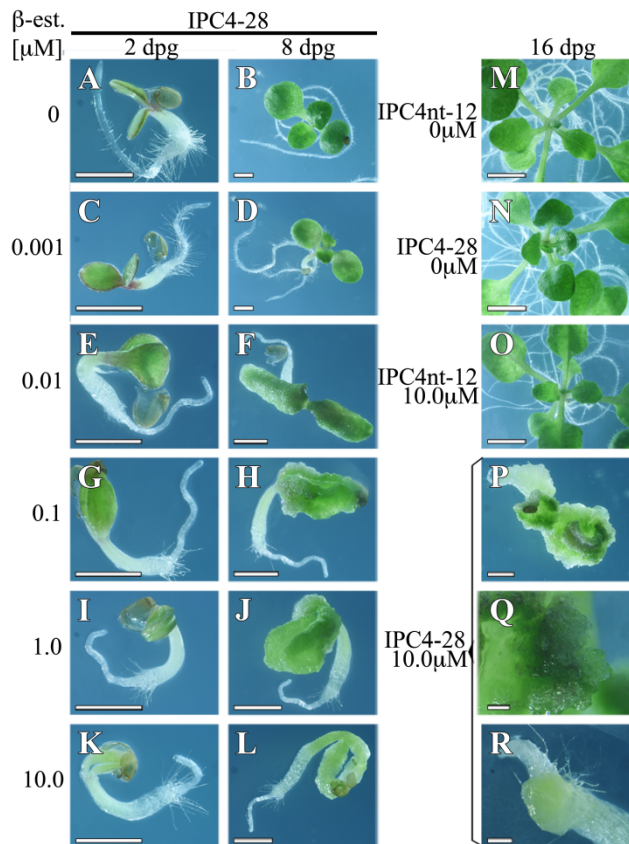


Fig. 3.2 Increasing concentrations of C4 protein correlate with phenotype severity in germinating seedlings. Images taken with a stereoscope show IPC4-28 seedlings germinated on solid media with varying concentrations of β -estradiol (0 to 10 μ M) at 2 days post germination (dpg) **(A, C, E, G, I, K)**, 8 dpg **(B, D, F, H, J, L)**, and 16 dpg **(M, N, O, P, Q, R)**. Control non-induced **(M)** and induced **(O)** IPC4nt-12, and non-induced IPC4-28 **(N)** seedlings at 16 dpg. Scale bar = 1mm **(A-L, and P)**, 3mm **(M-O)**, 200 μ m **(Q and R)**. β -est., β -estradiol

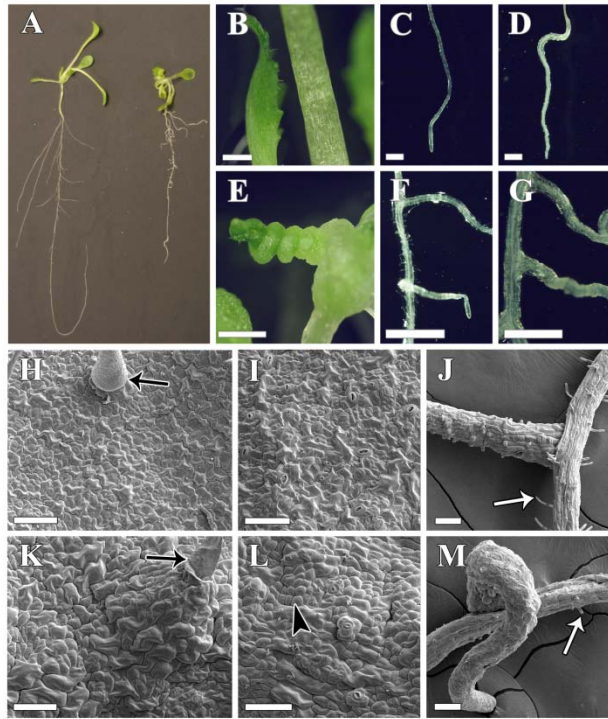


Fig. 3.3 Abnormal development of IPC4-28 seedlings expressing C4 postgermination. IPC4-28 seedlings were germinated in liquid media and allowed to grow for 7 days prior to the addition of 10 μM β -estradiol and observed at 48 hpi. (A) Seedlings grown in the absence of inducer (left) and presence of inducer (right). Close-up of root tips of non-induced (C) and induced (D) seedlings, and lateral roots of non-induced (F) and induced (G) seedlings. Third rosette leaf of non-induced (B) and induced (E) seedlings, the latter showing distinct corkscrewing. Scanning electron micrographs of the adaxial surface (H and K) and abaxial surface (I and L) of the first pair of rosette leaves, and the seventh lateral root (J and M) of non-induced (H-J) and induced (K-M) seedlings. Black arrow, trichomes; white arrow, root hairs; black arrowhead, smaller cell clusters. Scale bar =

500 μm (B-G), 50 μm (H, I, K, L), and 100 μm (J and M)

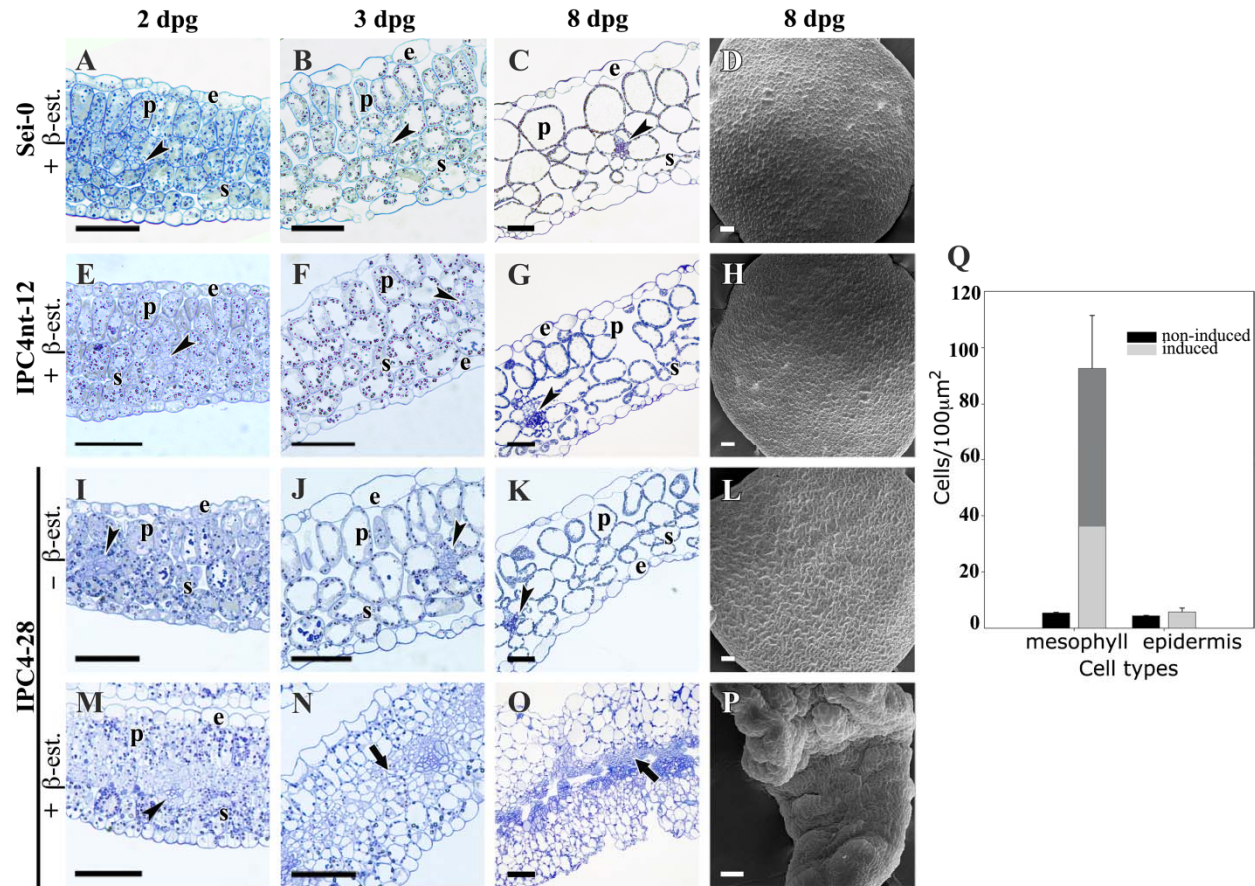


Fig. 3.4. *In planta* expression of C4 results in extensive cell division in cotyledons of *Arabidopsis*. Light microscopy analysis of cross-sections of cotyledons taken from seedlings at 2, 3, or 8 days postgermination (dpg) in the presence or absence of 1.0 μM β -estradiol. Cotyledon development of Sei-0 (A-C) and IPC4nt-12 (E-G) seedlings germinated in the presence of inducer, and IPC4-28 seedlings germinated in the absence (I-K) and presence (M-O) of inducer. Scanning electron micrographs of Sei-0 (D) and IPC4nt-12 (H) germinated in the presence of inducer and IPC4-28 germinated in the absence (L) and presence (P) of inducer. (Q) Cell density of epidermal and mesophyll cells from cross-sections of cotyledons taken from IPC4-28 seedlings at 8 dpg in the presence and absence of 1.0 μM β -estradiol. Cell density data represent means \pm SD for three independent cotyledon cross-sections from induced and non-induced seedlings. Mesophyll cell number from cotyledons of induced seedlings represented the sum of the cell number from the layer of small cells in the middle of the cotyledon lamina (dark grey) plus the number of cells outside the middle lamina area (grey). β -est., β -estradiol. e, epidermis; p, palisade mesophyll; s, spongy mesophyll; arrow head, vascular bundles; arrow, densely packed cell layer towards middle of cotyledon lamina. Scale bars = 50 μm (A-C, E-G, I-K and M-O), and 100 μm (D, H, L and P)

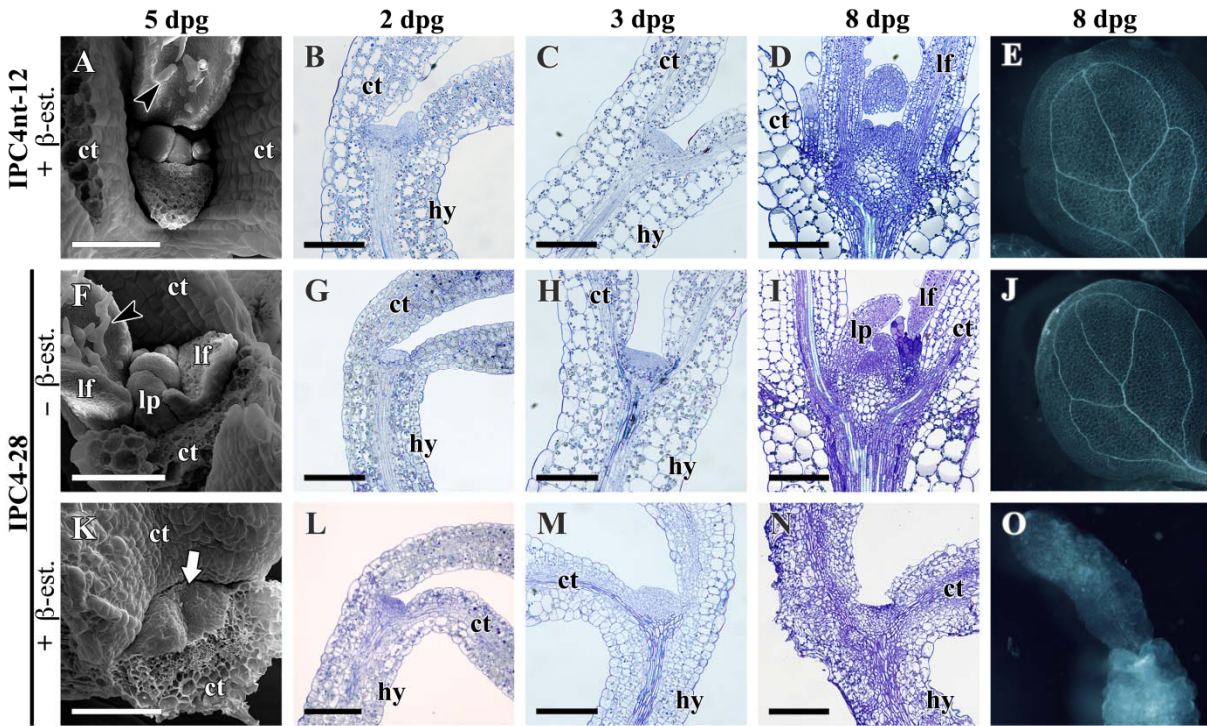


Fig. 3.5 *In planta* expression of C4 leads to loss of shoot apical meristem (SAM) and tissue organization. Scanning electron micrographs of IPC4nt-12 germinated in the presence of 10 μ M β -estradiol (A) and IPC-28 seedlings germinated in the absence (F) and presence (K) of inducer. Light micrographs of longitudinal sections through the SAM taken from control IPC4nt-12 seedlings germinated on media with 1.0 μ M inducer (B-D) and IPC4-28 seedlings germinated on media without (G-I) or with 1.0 μ M inducer (L-N). Cleared cotyledons of induced IPC4nt-12 seedlings (E), and of non-induced (J) and induced (O) IPC4-28 seedlings. β -est., β -estradiol; ct, cotyledon; hy, hypocotyl; lf, leaf; lp, leaf primordia; black arrowhead, trichomes; white arrow, stunted lp and SAM. Scale bars = 100 μ m

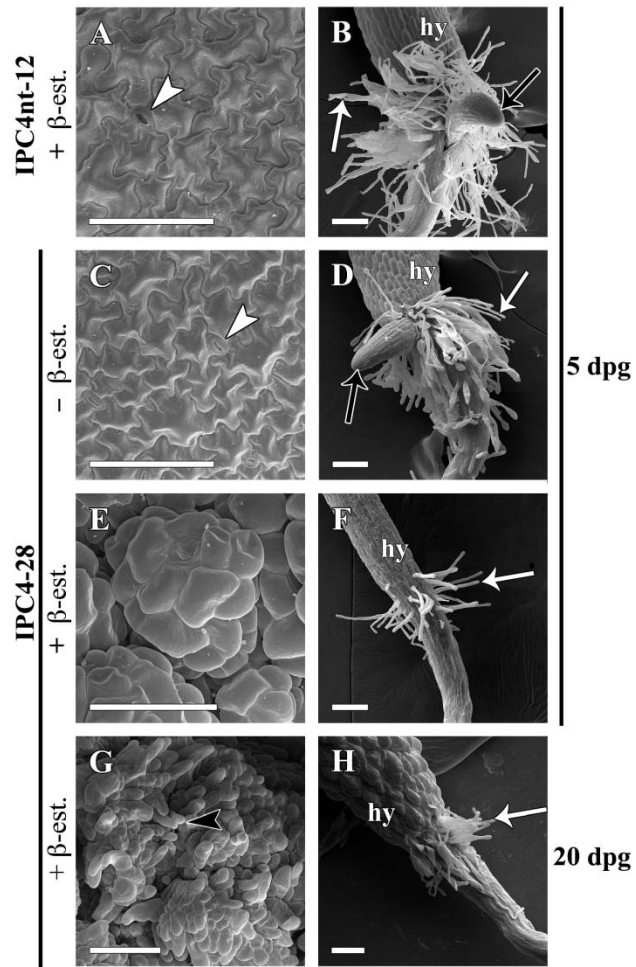


Fig. 3.6 *In planta* expression of C4 leads to loss of lateral root and stomata development, abnormal epidermal cell patterning, and reduced root hair formation. Scanning electron micrographs of IPC4nt-12 control seedlings germinated on solid media in the presence of 10 μ M β -estradiol (**A** and **B**) and IPC4-28 seedlings germinated in the absence of inducer (**C** and **D**) at 5 dpg, and IPC4-28 seedlings germinated in the presence of inducer at 5 dpg (**E** and **F**) and 20 dpg (**G** and **H**). (**A**, **C**, **E**, and **G**) Abaxial epidermis of cotyledons. (**B**, **D**, **F**, and **H**) Hypocotyl/root transition zone. β -est., β -estradiol; hy, hypocotyl; black arrow, lateral roots; black arrowhead, finger-like epidermal cells; white arrow, root hairs; white arrowhead, stomata. Scale bars = 100 μ m

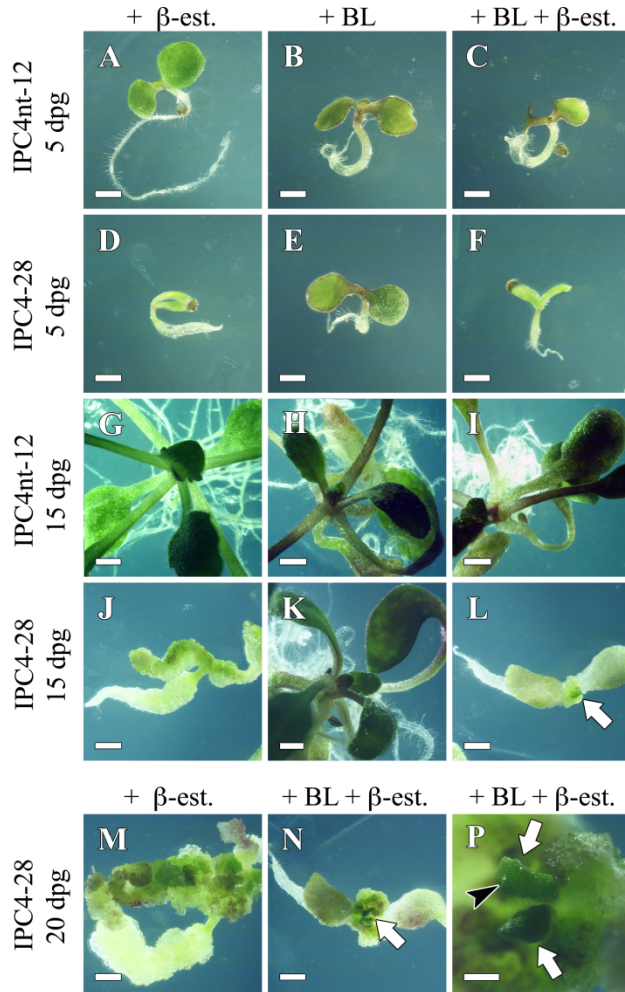


Fig. 3.7 BR partially rescues the C4-expression phenotype. Seedlings were germinated on solid induction media (**A, D, G, J, and M**), on solid media with 1 μ M BL (**B, E, H, and K**), or solid induction media amended with 1 μ M BL (**C, F, I, L, N, and P**). Induction media contained 10 μ M β -est. Seedlings were observed at 5 dpg (**A-F**), 15 dpg (**G-L**), and 20 dpg (**M-P**). β -est., β -estradiol; BL, brassinolide; dpg, days postgermination; white arrow, putative leaf primordia; black arrowhead, trichome. Scale bar = 1000 μ m (**A-N**), 250 μ m (**P**)

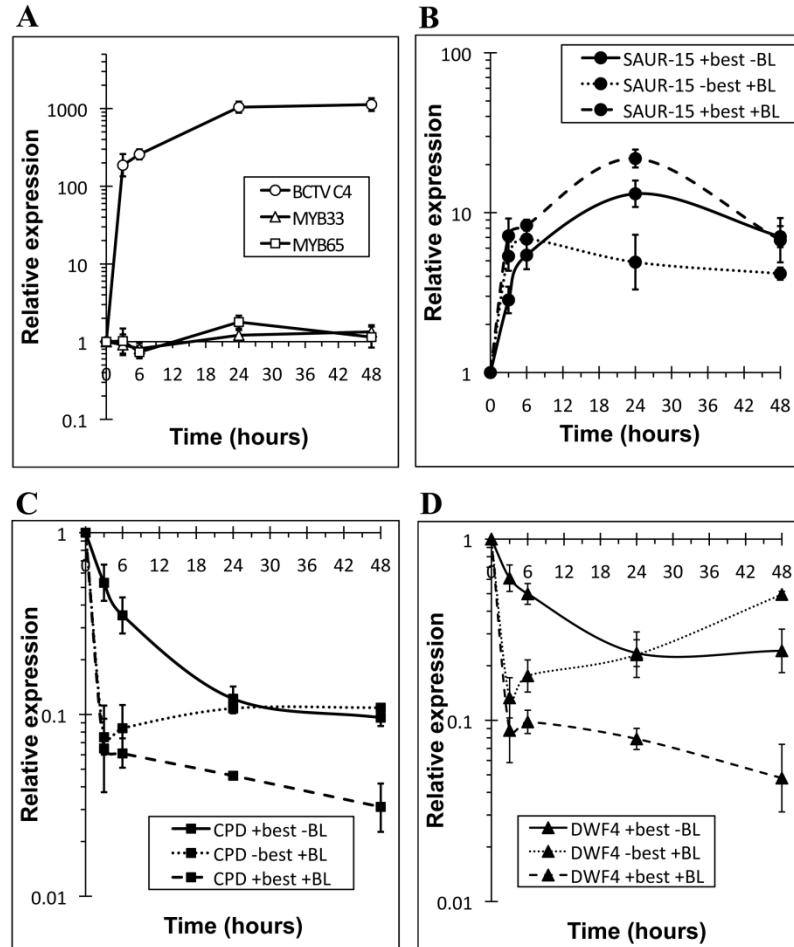


Fig. 3.8 Quantitative real-time PCR analysis. IPC4-28 seedlings were germinated for 7 days in liquid media then treated with 10 μ M β -estradiol, 1.0 μ M BL, or 10 μ M β -estradiol and 1.0 μ M BL. Control seedlings were untreated. Seedlings were collected at 3, 6, 24, 48 and 96 hpi and total RNA was extracted and assayed by qRT-PCR. Relative expression levels of C4 and miRNA regulated MYB genes, *MYB33*, and *MYB65* (A), and BR-regulated genes *SAUR-15* (B), *CPD* (C), and, *DWF4* (D). Solid lines indicate treatment with 10 μ M β -estradiol only, dotted lines indicate treatment with 1.0 μ M BL only, and dashed lines indicate treatment with both 10 μ M β -estradiol and 1.0 μ M BL. Relative transcript abundance for each gene was normalized to *ACT2*. Data represents means \pm SD for three independent biological replicates. best., β -estradiol; BL, brassinolide

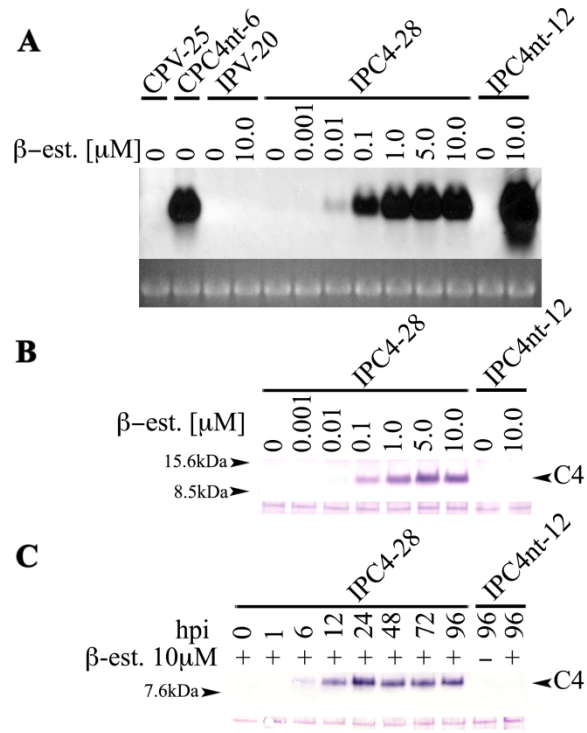


Figure S 3.1 Expression of C4 mRNA and protein correlates with inducer concentrations and time following induction. **(A)** Northern blot analysis on total RNA extracted from seedlings germinated in liquid for 7 days prior to induction with varying concentrations of β -estradiol (0 to 10 μ M) and collected 24 hours post-induction (hpi) (upper panel). Transgenic lines IPC4-28, IPC4nt-12, and IPV-20 express wild type C4, a non-translatable version of C4 (C4nt), and empty vector (V) from a β -estradiol inducible promoter (IP), respectively. Control lines CPC4nt-6 and CPV-25 express C4nt and empty vector from the constitutive 35S promoter (CP), respectively. rRNA loading control is indicated in the lower panel. **(B)** Western blot analysis of total protein extracted from seedlings treated as described in **(A)**. Non-specific protein band used as loading control is indicated in the lower panel. **(C)** Western blot analysis of total protein extracted from seedlings germinated and grown in liquid for 7 days prior to induction with 10 μ M β -estradiol and collected at various times after induction (upper panel). Non-specific protein band used as loading control indicated in the lower panel. Protein molecular mass markers and C4 are indicated in **(B)** and **(C)**. β -est., β -estradiol

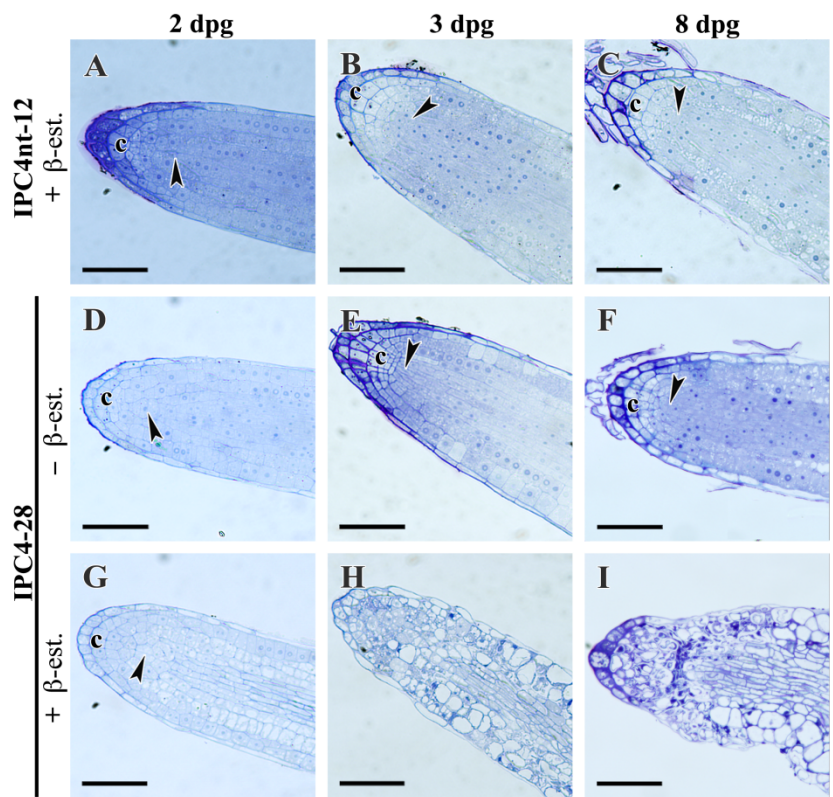


Figure S 3.2 *In planta* expression of C4 leads to loss of root apical meristem (RAM) organization. Longitudinal sections of RAMs of IPC4nt-12 seedlings germinated on media with 1.0 μ M β -estradiol (A-C) and IPC4-28 seedlings germinated on media without inducer (D-F) and on media with 1.0 μ M inducer (G-I), analyzed by light microscopy. Samples were taken from seedlings at 2, 3, and 8 dp. β -est., β -estradiol; c, columella; black arrow head, quiescent center. Scale bars = 50 μ m

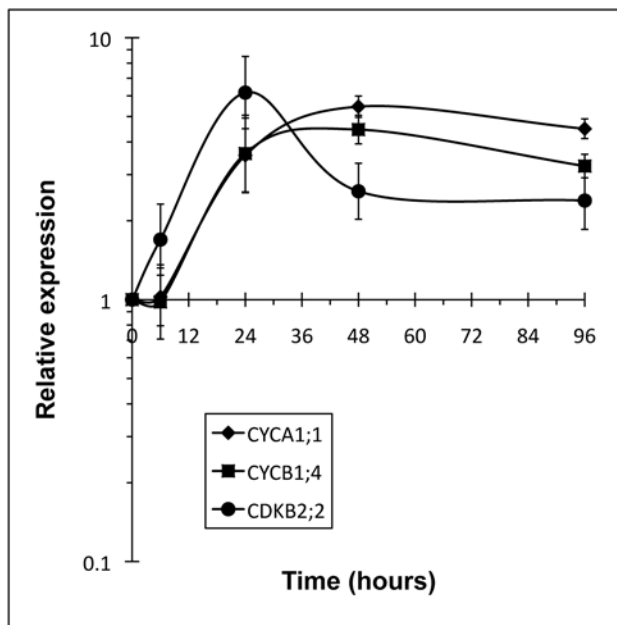


Figure S 3.3 Quantitative real-time PCR analysis of *CYCA1;1*, *CYCB1;4*, and *CDKB2;2* mitotic markers. IPC4-28 seedlings were germinated for 7 days in liquid media then treated with 0.0 or 10 μ M β -estradiol. Seedlings were collected at 3, 6, 24, 48 and 96 hpi and total RNA was extracted and assayed by qRT-PCR. The relative expression levels of each gene are indicated. Relative transcript abundance for each gene was normalized to *ACT2*. Data represents means \pm SD for three independent biological replicates

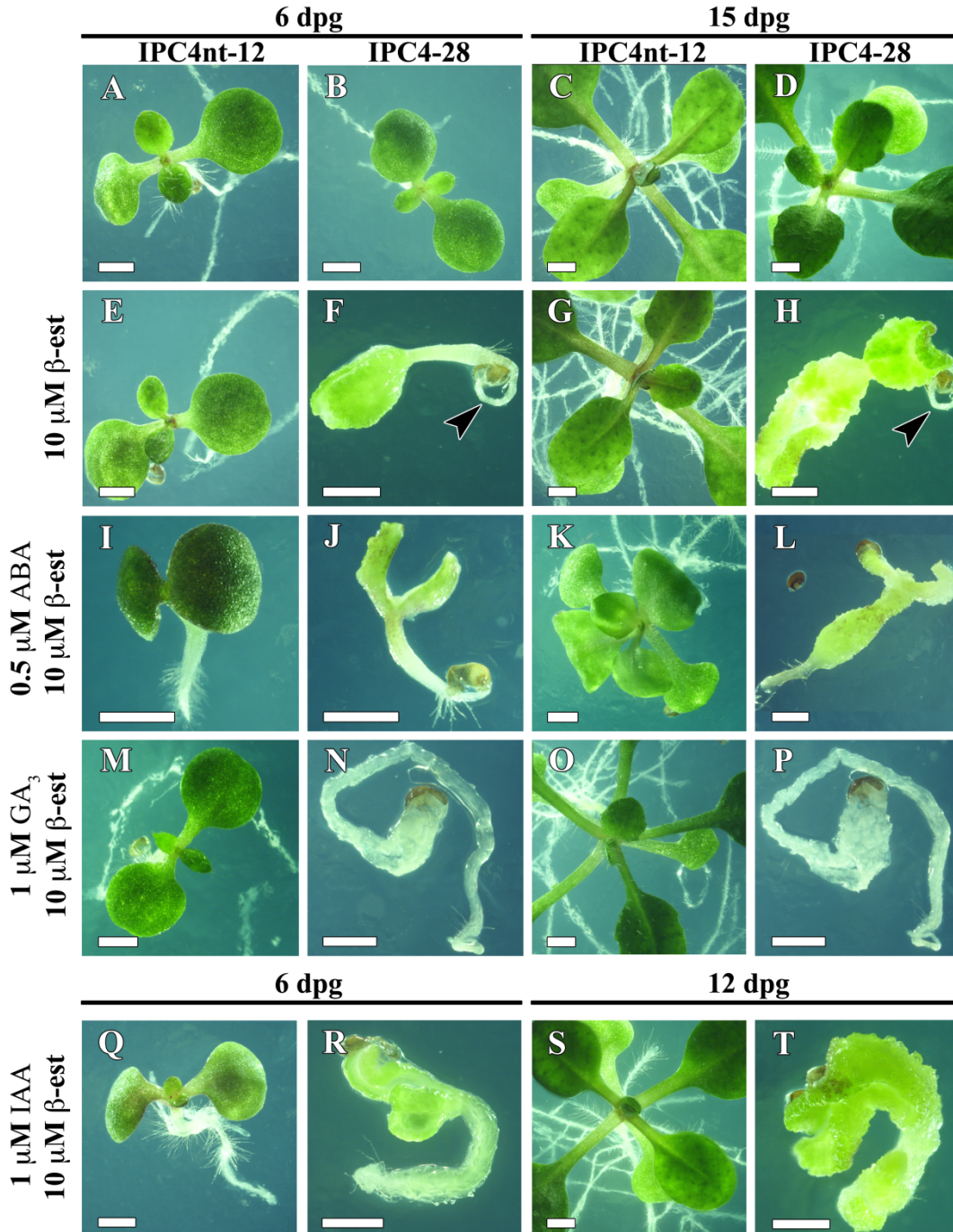


Figure S 3.4 Effects of hormone treatments on C4-induced phenotype. Seedlings were germinated on solid media (**A-D**), on solid induction media (**E-H**), on solid induction media amended with 0.5 μ M ABA (**I-L**), 1 μ M GA₃ (**M-P**) or 1 μ M IAA (**Q-T**). Induction media contained 10 μ M β -est. Seedlings were observed at 6 dpg and 15 dpg (**A-P**), or 6 dpg and 12 dpg (**Q-T**). Arrowhead, root; β -est., β -estradiol; ABA, abscisic acid; GA₃, gibberellic acid; IAA, the indole-3-acetic acid. Scale bar = 1000 μ m

CHAPTER 4

DISRUPTION OF THE BRASSINOSTEROID SIGNALING PATHWAY BY

GEMINIVIRUS C4 PROTEIN²

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Abstract

The C4 gene of *Beet curly top virus* (BCTV-B[US:Logan:76]) has been shown to induce hyperplasia and alter *Arabidopsis* development. Here we show that C4 interacts, *in planta*, with three closely related members of the *Arabidopsis* SHAGGY-like protein kinase (AtSK21, AtSK22 and AtSK23) family, which are negative regulators of brassinosteroid (BR) hormone signaling. BES1 and BZR1 transcription factors are substrates of AtSK21 kinase activity, and are inhibited when hyperphosphorylated. The C4 protein inhibits AtSK21 kinase activity on BES1, as expression of C4 leads to accumulation of dephosphorylated BES1, and changes in BES1/BZR1 target gene expression. A putative phosphorylation site (Ser at 49) on C4 was found to be required for the C4/AtSK interaction. Mutation of Ser49 to Ala abolished the C4/AtSK interaction, loss of the C4-expression phenotype, and failure to induce changes in the BR signaling pathway. Substitution of Ser49 by Thr restored both the C4/AtSK interaction and the effects of C4 on plant development and BR signaling. An active kinase activity is also required for the interaction, since AtSK kinase dead mutants did not interact with C4. These results suggest that C4 is a substrate for subgroup 2 AtSK kinases, resulting in the positive induction of the BR signaling pathway downstream of AtSK activity, and activation of the C4 protein.

Introduction

The family *Geminiviridae* is divided into four genera based on genome organization, host range and insect vector: *Begomoviruses*, which can be bipartite or monopartite, *Curtoviruses*, *Mastreviruses* and *Topocuviruses*. All geminiviruses express the C4 protein, designated AC4 protein in bipartite begomoviruses, except for mastreviruses. The small C4/AC4 protein has been implicated in many different aspects of viral infection and pathogenicity, such as movement (Rojas et al., 2001), regulation of plant development (Chellappan et al., 2004; Mills-Lujan and Deom, 2010), and regulation of cell cycle control (Lai et al., 2009; Mills-Lujan and Deom, 2010). The C4 gene of *Beet curly top virus* (BCTV-B[US:Logan:76]; referred to hereafter as BCTV) has been shown to induce hyperplasia in plants (Stanley and Latham, 1992; Latham et al., 1997) and more recently to increase cell proliferation and alter the development of transgenic *Arabidopsis thaliana* plants (Mills-Lujan and Deom, 2010).

The mechanism behind the function of the BCTV C4 protein (designated C4 hereafter) is still not well understood. Evidence suggests that C4 interacts with and interferes with the brassinosteroid (BR) signaling pathway (Piroux et al., 2007; Mills-Lujan and Deom, 2010). C4 was shown to interact with two members of the *Arabidopsis* SHAGGY-like protein kinase family (AtSK), AtSK21 and AtSK23, and a leucine-rich repeat receptor-like kinase (LRR-RLK) of unknown function in a yeast two-hybrid assay. Moreover C4 was shown to be a substrate for AtK21 in an *in vitro* phosphorylation assay, suggesting a regulatory role for phosphorylation in C4 function (Piroux et al., 2007). More recently experiments showed the C4 protein altered the expression profiles of BR signaling pathway target genes in transgenic *A. thaliana* plants (Mills-Lujan and Deom, 2010).

AtSKs, encoded by a multigene family (ten members) of non-receptor serine/threonine protein kinases, are involved in diverse cellular processes including hormone signaling, development, and stress responses (Jonak and Hirt, 2002). Based on the sequence comparison of the catalytic domains the AtSK gene family was divided into four subgroups (Dornelas et al., 1998; Yoo et al., 2006). Subgroup 2 AtSKs (sg2 AtSKs; AtSK21, AtSK22, and AtSK23) are important negative regulators of BR hormone signaling. In the current model of the BR signaling pathway sg2 AtSKs are active in the absence of the BR signal (Ryu et al., 2007; Yan et al., 2009) hyperphosphorylating two closely related plant-specific transcription factors, BZR1 and BES1 (Belkhadir and Chory, 2006), leading to their inactivation (He et al., 2002; Yin et al., 2002; Vert and Chory, 2006; Gampala et al., 2007; Ryu et al., 2007). However, in the presence of BR the hormone binds to the BRI1 receptor kinase (Li and Chory, 1997; Kinoshita et al., 2005) and induces dissociation of BKI1, an inhibitor of BRI1. Subsequently, BRI1 and its co-receptor BAK1 become activated (Wang and Chory, 2006) and the plasma membrane receptor-complex phosphorylates BSK1 (Tang et al., 2008), allowing it to interact with, and presumably activate BSU1 phosphatase (Kim et al., 2009). BSU1 dephosphorylates AtSK21, and possibly other AtSKs involved in BR signaling, thereby inactivating the kinases, and resulting in the accumulation of hypophosphorylated BZR1 and BES1 (Kim et al., 2009). Since BZR1 is a repressor, its target genes, such as *CPD*, are down-regulated in the presence of BR (He et al., 2005); whereas, BES1 is an activator that induces the expression of genes, such as *SAUR-15* (Yin et al., 2005). Recent evidence suggests that additional AtSKs are also involved in regulating the BR signaling pathway (De Rybel et al., 2009; Kim et al., 2009).

The C4 protein was previously shown to interact *in vitro* with AtSK21 and AtSK23 (Piroux et al., 2007), but the biological significance of this interaction has not been established.

Herein we show that C4 interacts specifically with all sg2 AtSKs *in planta*. In addition, amino acid residues were identified on C4 and the sg2 AtSKs that are important for the interaction. Adequate localization of the C4/sg2 AtSK interaction to the plasma membrane resulted in inhibition of sg2 AtSKs function in BR signaling.

Results

Serine residue at position 49 is important for C4 function

We recently showed that the ectopic expression of the C4 gene in *Arabidopsis thaliana* led to abnormal plant development (Mills-Lujan and Deom, 2010). To further investigate the function of the C4 gene, alanine-scanning mutagenesis (Cunningham and Wells, 1989) was performed on amino acids residues that are highly conserved between the C4 proteins of four closely related curtoviruses (BCTV, *Beet mild curly top virus* (BMCTV-[US:Wor]), *Beet severe curly top virus* (BSCTV-[US:Cfh]), and *Spinach curly top virus* (SpCTV-[US:Sp3:36])) and two monopartite begomoviruses (*Tomato yellow leaf curl virus* (TYLCV-IL[ES:Alm:Pep:99]), and *Tomato leaf curl virus* (ToLCV-To[AU])); Fig. 4.1A). C4 mutants were screened for phenotype on *Nicotiana benthamiana* using a PVX-based vector (Chapman et al., 1992). A non-translatable version of the C4 gene (C4nt) and the C4G2A mutant with a Gly to Ala mutation at the second amino acid residue, previously shown to be important for myristoylation and function of C4 (Fondong et al., 2007; Piroux et al., 2007), were used as negative controls (Fig. 4.1B). Plant inoculated with PVX-C4nt, PVX-C4G2A or empty PVX vector were indistinguishable from each other and showed a mild mottling phenotype (Fig. 4.1B). Initial mutagenesis targeted six

conserved basic residues, five conserved acidic residues and three double substitutions of charged residues (Table 4-2). Only the Lys to Ala mutation at residue 13 (C4K13A) had a mild C4-like phenotype (Table 4-2 and Figure S 4.1). All other charged amino acid mutants retained a C4-like phenotype characterized by rolling petioles, and epinastic, rough, bumpy and chlorotic leaves (Table 4-2 and Figure S 4.1). In addition, a non-sense mutation introduced at residue 72 (C4-72Stp) abolished C4 function (Table 4-2 and Figure S 4.1), whereas a non-sense mutation introduced at residue 83 (C4-83Stp) had no effect on the C4-phenotype (Table 4-2).

Since C4 is phosphorylated *in vitro* by AtSK21 (Piroux et al., 2007), mutagenesis was performed on eight conserved Ser residues and four conserved Thr residues (Table 4-2) to identify a putative phosphorylation site(s) required for function (Fig. 4.1A). Only the Ala substitution at residue 49 (C4S49A) induced a PVX-like phenotype (Fig. 4.1B), all other Ser and Thr mutants induced a C4-like phenotype on inoculated *N. benthamiana* (Table 4-2). If phosphorylation of Ser49 is required for function, then Thr, which can substitute for Ser as a substrate for phosphorylation, could restore the C4 phenotype. When Thr was substituted for Ala in the C4S49A mutant, *N. benthamiana* plants inoculated with PVX-C4S49T developed a C4-like phenotype (Fig. 4.1B), indicating that Thr can rescue the C4 phenotype. In some cases, Glu or Asp residues can function as phospho-mimics, therefore we substituted Glu for Ser49 (C4S49E). PVX-C4S49E inoculated plants developed a PVX-like phenotype (Table 4-2), indicating that Glu phosphorylation mimicking does not rescue the phenotype induced by Ser49 or Thr49 .

To further confirm the loss of function of the C4S49A mutation we generated transgenic *A. thaliana* plants with C4S49A under the regulatory control of the β -estradiol inducible promoter (Zuo et al., 2000) as previously described in characterizing the effects of C4 on *A.*

thaliana development (Mills-Lujan and Deom, 2010). A single copy insertion transgenic line, IPC4S49A-2, was chosen for further study. In addition, we generated transgenic *Arabidopsis* plants with the C4S49T mutant under the control of the β -estradiol inducible promoter (lines IPC4S49T-2 and IPC4S49T-6). The phenotype of the resulting lines was compared to transgenic lines IPC4-28 and IPC4-18, which express wild type C4, and line IPC4nt-12, which expresses a non-translatable version of C4. Seedlings of the six lines developed normally and were indistinguishable from wild-type *Arabidopsis* when germinated in the absence of the β -estradiol inducer (Fig. 4.2A, C, E, G, I and K). As previously shown, germination on induction media leads to severe stunting and hyperplasia of line IPC4-28, but has no effect on line IPC4nt-12 (Mills-Lujan and Deom, 2010; Fig. 4.2B and L, respectively). The expression of C4 also induces hyperplasia and stunting in line IPC4-18, although the phenotype is slightly less severe than that induced by IPC4-28 (Fig. 4.2B and D), possibly due to positional effects of the transgene insertion site. Seedlings of line IPC4S49A-2 germinated on induction media showed a phenotype that was indistinguishable from non-induced seedlings (Fig. 4.2I and J), suggesting a loss of C4 function. Induced IPC4S49A-2 seedlings showed some anthocyanin accumulation, which is indicative of stress, and could be due to the high levels of C4S49A protein accumulation (Fig. 4.5A). Both IPC4S49T-2 and IPC4S49T-6 seedlings showed a phenotype similar to that of IPC4-28 and IPC4-18, respectively, when germinated in the presence of the inducer (Fig. 4.2F and H). This indicates that the C4S49A mutation abolishes the function of the C4 protein in *Arabidopsis*, and that the C4S49T mutation rescues the C4 phenotype.

Both a functional kinase and a putative phosphorylation site on C4 are required for the interaction between C4 and subgroup 2 AtSKs in vitro.

The C4 protein was shown to interact with AtSK21 and AtSK23 in a yeast two-hybrid assay (Piroux et al., 2007). The three sg2 AtSKs have been implicated in regulating the BR signaling pathway (Ryu et al., 2007) and, more recently, other AtSK family members have also been implicated in BR signaling (De Rybel et al., 2009; Yan et al., 2009). To investigate whether C4 interacts with other AtSK family members, we performed a yeast two-hybrid assay and quantified the relative β -galactosidase activity to determine the strength of the C4/AtSK interactions. C4 interacted with all sg2 AtSKs (Fig. 4.3A), with the interaction to AtSK23 being the strongest. However, C4 failed to interact, or interacted weakly, with the other seven AtSK family members (Fig. 4.3A; sg1 AtSK11, -12, -13; sg3 AtSK31, -31; sg4 AtSK41, -42).

We also tested if the interaction between C4 and the AtSKs was altered by the loss of function mutations, C4G2A and C4S49A. There was no change in the strength of the interaction between the C4G2A mutant and sg2 AtSKs, when compared to the wild type C4/AtSK interactions (Fig. 4.3C). However, the C4S49A mutation completely abolished the interaction with sg2 AtSKs (Fig. 4.3C), whereas the reversion mutant (C4S49T) interacted as well as the wild type C4 with sg2 AtSKs (Fig. 4.3C). This suggests that a phosphate-accepting residue at position 49 is important for the interaction between C4 and sg2 AtSKs.

To further confirm the importance of a putative phosphorylation site for C4/AtSK interactions, we determined if sg2 AtSK kinase dead mutants were also able to interact with C4. A kinase-dead (KD) mutant of AtSK21, AtSK21K69R (referred to hereafter as AtSK21-KD), was unable to phosphorylate BZR1 (Zhao et al., 2002; Ryu et al., 2007). The KD mutant of

AtSK21 and the homologous mutants in AtSK22 and AtSK23 (AtSK22K99R and AtSK23K101R, referred to hereafter as AtSK22-KD and AtSK23-KD, respectively) did not interact with C4 (Fig. 4.3B). To confirm that the loss of interaction was site-specific, gain-of-function mutants (GF), which have increased protein stability (Li and Nam, 2002; Peng et al., 2008; Yan et al., 2009), were used. The GF mutation had no effect on the C4/AtSK interactions (Fig. 4.3C). These results indicate that the loss of the C4/AtSK interactions was specific to the lack of a functional kinase domain on the sg2 AtSKs. Taken together the results suggest that both an active kinase domain on sg2 AtSKs, and a functional phosphorylation acceptor site on C4 are important for the C4/AtSK interaction.

C4 and subgroup 2 AtSKs interact in planta

To determine if the interactions seen in the yeast two-hybrid assay also occurred *in planta*, bimolecular fluorescence complementation (BiFC) assays (Citovsky et al., 2006) were conducted. Initially, C4 was tagged with either the N-terminal portion of enhanced yellow fluorescent protein (nEYFP; C4-nEYFP) or the C-terminal portion of EYFP (C4-cEYFP) and cloned into the PVX-based vector to assess the functionality of the fusion proteins in *N. benthamiana*. The phenotype induced by the C4-cEYFP fusion protein was similar to that induced by the wild type C4 protein on inoculated plants (Figure S 4.1B), while the C4-nEYFP fusion induced a less severe phenotype (Figure S 4.1B). Therefore, the C4-cEYFP fusion protein was used in combination with nEYFP fused to AtSKs (AtSKs-nEYFP) for the BiFC assays. The C4-cEYFP and individual AtSK-nEYFP fusion proteins were co-agro-infiltrated into transgenic *N. benthamiana* plant line CFP-H2B which constitutively expresses a cyan fluorescent protein

(CFP) fused to histone 2B that localizes to the nucleus (Martin et al., 2009) and can thus serve as a spatial reference. Sonchus yellow net virus (SYNV) N protein fused to nEYFP and SYNV P protein fused to cEYFP served as a positive control (Martin et al., 2009). The EYFP signal resulting from the N/P interaction localized to the nucleus (Martin et al., 2009) and co-localized with the CFP-H2B reference signal (Fig. 4.4A). As a negative control we used nEYFP fused to the N-terminus of GST in combination with C4-cEYFP. As expected, no C4/GST interaction was detected in inoculated *N. benthamiana*, but the CFP signal was visible in the nucleus (Fig. 4.4A).

When C4-cEYFP was co-infiltrated into *N. benthamiana* plants in combination with different AtSK-nEYFP fusions, C4-cEYFP interacted with sg2 AtSKs (Fig. 4.4A and B). The C4/sg2 AtSK interactions occurred primarily at the plasma membrane, with some EYFP signal also detectable in the nucleus. This nuclear signal is clearly evident in the overlay between the EYFP and CFP signals. This is in contrast with the primarily cytoplasmic and nuclear localization of sg2 AtSKs, fused to intact mCherry fluorescent protein, when infiltrated alone (Figure S 4.3B), indicating that C4 changes the localization pattern of sg2 AtSKs. No interaction was detected between C4 and AtSK12, AtSK13, or AtSK41 (Fig. 4.4C), confirming the yeast two-hybrid results (Fig. 4.3) and indicating that C4 interacts specifically with sg2 AtSKs *in planta*. In addition, the interaction between the C4 mutants, C4S49A and C4S49T, and the sg2 AtSKs was determined. First, C4, C4S49A and the C4G2A control were expressed as fusions to intact EYFP. C4S49A-EYFP localized to the plasma membrane, as did the wild type C4-EYFP; additionally, C4-EYFP also localized to the nucleus (Figure S 4.3A). As expected, C4G2A-EYFP did not target to the plasma membrane, but instead was found in the nucleus and cytoplasm, as evidenced by the cytoplasmic strands (Figure S 4.3A; Piroux et al., 2007). As

expected from the yeast two-hybrid assays, the C4S49A mutant does not interact with sg2 AtSKs (Fig. 4.4B). However, the reversion mutant C4S49T did interact with sg2 AtSKs and localized to the plasma membrane and nucleus, in pattern that was indistinguishable from C4/sg2 AtSK interactions (Fig. 4.4B). This indicates that a phosphate-accepting residue is required for the interaction between C4 and sg2 AtSKs. Further support for a phosphate-accepting residue at Ser49 comes from assays in which we determined if C4 could interact with the sg2 AtSK-KD mutants *in planta*. The EYFP signal from C4/AtSK21-KD and C4/AtSK23-KD complexes were barely detectable (Fig. 4.4B), indicating that the interactions were very weak, and suggesting that a functional kinase activity is important for the C4/sg2 AtSK interactions.

The C4G2A mutant also interacts with sg2 AtSKs, and results indicated it appears to interact more strongly than wild type C4 (Figure S 4.2) as shown by EYFP signal intensity. However, the EYFP signal resulting from the C4G2A/sg2 AtSK interactions was not localized to the plasma membrane, but rather was dispersed throughout the cytoplasm and nucleus. This was confirmed by overlaying the autofluorescence (AF) of chloroplasts onto the EYFP signal from the C4G2A/sg2 AtSK interactions. Unlike with C4/sg2 AtSK interactions where the EYFP signal and chloroplast AF were in separate cellular compartments, in the C4G2A/sg2 AtSK interactions there was clearly an overlap between the EYFP and chloroplast AF signals (Figure S 4.2). This confirms previous work that indicates that C4 has a functional N-myristoylation motif that targets the protein to the plasma membrane (Piroux et al., 2007), since the mutagenesis of the N-myristoylation motif in C4G2A results in the loss of membrane localization. Even though the interaction between sg2 AtSKs and C4G2A appears to be stronger than with wild type C4, the C4G2A mutation abolishes C4 function (Fig. 4.1 and Fig. 4.2), suggesting that proper

localization of C4 to the plasma membrane is required for the C4/sg2 AtSK interaction to be biologically important.

C4 was also shown to interact with a partial clone of a leucine-rich repeat receptor-like kinase (LRR-RLK) of unknown function in yeast two-hybrid and in vitro binding assays (Piroux et al., 2007). To determine if this interaction also occurred *in planta*, C4-cEYFP was co-infiltrated in combination with LRR-RLK-nEYFP into *N. benthamiana* CFP-H2B plants. We were unable to detect an EYFP signal (Fig. 4.4C), suggesting that C4 and LRR-RLK likely do not interact *in planta*.

Non-functional C4 fails to alter the BR signaling pathway

The expression of BR signaling target genes is altered in the presence of C4, in a manner similar to that of BL application (Mills-Lujan and Deom, 2010). AtSK21, and presumably AtSK22 and AtSK23, hyperphosphorylate BES1 (pBES1 for brevity) in the absence of BR, whereas after BL treatment or in the absence of sg2 AtSKs the hypophosphorylated form of BES1 accumulates (Yin et al., 2002; Mora-Garcia et al., 2004; Yan et al., 2009). We hypothesized that C4 expressing seedlings should have decreased levels of pBES1, whereas in C4S49A expressing seedlings the levels of pBES/BES1 should be similar to those of non-induced seedlings, as C4S49A does not interact with sg2 AtSKs (Fig. 4.3 and Fig. 4.4). To confirm that disruption of sg2 AtSKs kinase activities by C4 alters the BR signaling pathway, the phosphorylation status of BES1, in the presence and absence of C4 and C4 mutants, was determined. The BES1 phosphorylation status was compared in induced and mock-induced transgenic seedlings. In the absence of inducer, all seedling lines show similar amounts of BES1

and pBES1 (Fig. 4.5A). However, in the presence of inducer line IPC4-28 and IPC4S49T-2 seedlings showed a decrease in pBES and a concomitant increase in BES1; the shift from pBES1 to BES1 was not detected in induced IPC4S49A-2 seedlings (Fig. 4.5A). This indicated that a putative phosphate acceptor at residue 49 must be present on C4 in order for the shift in phosphorylation status of BES1 to occur.

Because the C4S49A mutant is not capable of binding to sg2 AtSKs (Fig. 4.4) and does not alter the BR signaling pathway response in a manner similar to wild type C4 (Fig. 4.5A), C4 may be competing as a phosphorylation substrate with BES1. To test this, IPC4-28 seedlings were induced with increasing concentrations of β -estradiol, which is positively correlated with the amounts of C4 expression (Mills-Lujan and Deom, 2010), and compared the pBES1/BES1 motility by western blot analysis. A slight increase in the accumulation of hypophosphorylated BES1 was detected even at the lowest concentration of β -estradiol used (0.01 μ M; Fig. 4.5B). The level of BES1 was augmented even more with an increasing concentration of inducer (0.1, 1.0 and 10.0 μ M β -estradiol), while the level of pBES1 dropped to undetectable levels at an inducer concentration of 1.0 μ M (Fig. 4.5B). This indicates that inhibition of sg2 AtSK kinase activity is dependent on the concentration of the C4 protein.

Discussion

It has been known for some time now that BCTV C4 protein induces cell division, but the mechanism behind the function of C4 remained unknown. This work demonstrates that C4 interacts specifically with subgroup 2 AtSKs, and that this interaction occurs *in planta* (Fig. 4.4). In addition, a Ser/Thr kinase phosphorylation acceptor residue at residue 49 in C4, as well as an

active kinase domain on sg2 AtSKs were shown to be required for the C4/sg2 AtSK interaction *in planta* (Fig. 4.4). This is supported by previous studies showing that AtSK21 phosphorylated C4 *in vitro* (Piroux et al., 2007). Finally, the loss of C4/sg2 AtSK interaction was shown to be biologically significant as it resulted in a loss of the C4-induced phenotype as well as the downstream effects on the BR signaling pathway (Fig. 4.5). Thus, we propose that C4 is phosphorylated by sg2 AtSKs and compete with the transcription factors that are normally targeted by these kinases. Inhibition of the kinase activity of host factors appears to be a common mechanism for geminivirus proteins. The closely related AL2 and L2 proteins suppress silencing and mitigate innate antiviral defense by inhibiting adenosine kinase (ADK) and SNF1 kinase, respectively (Hao et al., 2003; Yang et al., 2007). The nuclear shuttle protein (NSP) protein of bipartite begomoviruses, a multifunctional protein, was shown to interact with several plant host factors. NSP inhibits the activation of the plasma membrane receptor kinase NSP-interacting kinase (NIK), thereby blocking downstream events leading to a viral defense response (Fontes et al., 2004; Santos et al., 2009). The interaction between NSP and NSP-interactor (AtNSI), a nuclear acetylase, promotes viral DNA export from the nucleus (Carvalho et al., 2006). Phosphorylation of NSP by NSP-associated kinase (NsAK) appears to be a regulatory mechanism for NSP function (Florentino et al., 2006). Unlike bipartite begomoviruses, BCTV and other monopartite geminiviruses do not encode NSP. Therefore, other proteins in the monopartite geminivirus genome either substitute functionally for NSP or other geminivirus proteins have evolved to alternate functions that aid in the viruses' infection. Like NSP, C4 localizes to the plasma membrane and nucleus (Figure S 4.3), and appears to be a substrate for kinase activity (Fig. 4.3, Fig. 4.4, and Piroux et al., 2007). However, the mechanism behind the

function of C4 is distinct from that of NSP, since the host protein targets identified for each viral protein to date are different.

Geminivirus encoded proteins are multifunctional due to the limited coding capacity of the genome. It is conceivable that C4 protein interacts with additional host proteins. One possible candidate was the LRR-RLK (At3g24240) identified in yeast two-hybrid assays to interact with C4 (Piroux et al., 2007). However, an interaction between C4 and LRR-RLK was not detected *in planta* using BiFC (Fig. 4.4). Nevertheless, this does not exclude C4 protein interacting with other proteins. One possibility is that phosphorylated C4 protein (pC4) is required for downstream events. The phosphorylation of C4 could somehow activate the protein or induce a conformation change, allowing it to interact with other proteins. Alternatively, pC4 could be a substrate for additional host factors, such as phosphatases. The fact that Glu was not able to substitute for Ser49, whereas Thr was (Fig. 4.1 and Fig. 4.5), supports this idea. A second possibility is that the yeast two-hybrid screen missed possible interactors, either because of low library coverage or due to occlusion of host-protein binding sites on C4 by the large activation domain fusion protein. The latter is supported by our observation that the larger C4-nEYFP fusion protein induced a milder phenotype than C4-cEYFP (compare 19.8 kDa to 7.2 kDa for nEYFP and cEYFP, respectively), when inoculated onto *N. benthamiana* (Figure S 4.1). Thus, the use of a smaller, less disruptive tag on the small 9.4 kDa C4 protein could yield further host protein partners.

Regulation of BR signaling does not appear to be exclusive to sg2 AtSKs. Recent studies suggest that sg1 AtSKs and AtSK32 are also involved in BR signaling (De Rybel et al., 2009; Kim et al., 2009; Yan et al., 2009). We found that C4 interacted specifically with sg2 AtSKs (Fig. 4.3 and Fig. 4.4), and that this interaction was biologically significant. The expression of

C4 led to changes reminiscent of those that occur during BR-induction, in both the phosphorylation status of sg2 AtSK substrates, and in the expression of BR signaling pathway target genes (Fig. 4.5 and (Mills-Lujan and Deom, 2010)). Also, C4 induces a severe phenotype (Fig. 4.2), which was previously shown to be partially rescued by the addition of brassinolide (BL; the most biologically active BR; Mills-Lujan and Deom, 2010). This seemed counter-intuitive at the time, as the changes in *CPD*, *DWF4*, and *SAUR-15* expression induced by the presence of C4 or BL alone are similar. One would expect no additional effect or a synergistic effect of additional BL application to C4-expressing seedlings, which was not the case. Our recent finding that C4 interacts specifically with sg2 AtSKs (Fig. 4.4), and the apparent involvement of other AtSKs in BR signaling (De Rybel et al., 2009; Kim et al., 2009; Yan et al., 2009), indicates that the partial recovery seen with BL application to C4-expressing seedlings was likely due to a response by subgroup 1 AtSKs and AtSK32 to the BR. However, the C4-induced phenotype is more severe than the triple-knockout mutant for sg2 AtSKs (Vert and Chory, 2006; Yan et al., 2009; Mills-Lujan and Deom, 2010), raising the possibility that the effects of C4 on *Arabidopsis* development are not due solely to disruption of the BR signaling pathway. As previously suggested, C4 could interact with other host proteins directly or pC4 could be the substrate for downstream targets. Future studies aimed at discovering additional host proteins that interact with C4 will shed more light on the mechanism by which C4 functions.

Materials and Methods

Mutagenesis and mutant screen

The BCTV-B [US:Log:76] C4 gene was amplified by polymerase chain reaction (PCR), as described (Mills-Lujan and Deom, 2010), using primers PVXC45 and PVXC43. The PCR

product was cloned into pBSKS+ (Stratagene, La Jolla, CA) to give pBSKS-C4. Site-directed mutagenesis of the C4 gene was carried out using QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with pBSKS-C4 as a template and the primers listed in Table S 4-1, following the manufacturer's instructions. C4 mutants (Table 4-2) were excised using *Cla*I and *Eco*RV and subcloned into the PVX-based expression vector pP2C2S (Chapman et al., 1992) to give 40 different pP2C2S-C4 mutant constructs. A non-translatable version of the C4 gene (C4nt) was also cloned into pP2C2S, as above using primers PVXC45 and C4NTE103, to give pP2C2S-C4nt. The integrity of the C4, C4nt and C4mut genes in all plasmids was confirmed by sequencing.

RNA transcripts were obtained using RiboMax Large Scale RNA Transcription System T7 (Promega, Madison, WI) and *Spe*I linearized pP2C2S-C4, pP2C2S-C4nt or pP2C2S-C4 mutant expression vectors as a template, following manufacturer's instructions. Transcribed RNA was used to rub-inoculate *Nicotiana benthamiana* plants. Plants were kept in an environmentally controlled growth chamber (25°C, 16/8-h day/night cycles) and monitored for 14 days, the phenotype was noted, and tissue samples were collected.

Generation of Transgenic *Arabidopsis* Plant Lines

The C4S49A and C4S49T mutant genes were PCR-amplified, using primers LOGAN45 and LOGANC43, and pBSKS-C4S49A or pBSKS-C4S49T, as a template. The PCR products were cloned into pER10, as previously described (Mills-Lujan and Deom, 2010), to give pERC4S49A and pERC4S49T. Similarly, the C4G2A mutant gene was PCR-amplified using primers C4G2ApER5 and LOGANC43 and cloned into pER10 to give pERC4G2A. Genes cloned into pER10 are under regulatory control of an estradiol-inducible promoter (Zuo et al.,

2000). Plasmids were used for transforming *Arabidopsis thaliana* (*Arabidopsis*) ecotype Sei-0 and screened for single-copy insertion homozygotes, as previously described (Clough and Bent, 1998; Mills-Lujan and Deom, 2010) to obtain plant lines IPC4S49A-2, IPC4S49T-2 and IPC4S49T-6.

Yeast two-hybrid assay

The C4 gene was PCR amplified as described (Mills-Lujan and Deom, 2010), using primers baitC45PR and BDC43PR. The PCR product was digested with *Nco*I and *Bam*HI and cloned into the multiple cloning site of the pGADT7 or pGBKT7 vectors (Clontech, Mountain View, CA), which places C4 downstream of the GAL4 activation domain (pGADT7-C4) or the GAL4 binding domain (pGBKT7-C4), respectively. The C4 mutants were cloned in a similar manner, using pP2C2S-C4mut constructs as a template, except the C4 truncation mutant (C4-71Stp) was cloned using primers baitC45PR and 72StpBamHI, and the C4G2A mutant was cloned using primers C4G2A5Nco and BDC43PR. The ten AtSK gene family members were PCR amplified from *Arabidopsis* ecotype Sei-0 cDNA, using the primers indicated in Table 4-1, cloned into either pGEM-T (Promega, Madison, WI) or pSCA-amp/kan (Stratagene, La Jolla, CA), and then subcloned into the multiple cloning site of the pGADT7 or pGBKT7 vectors (Clontech, Mountain View, CA) using the appropriate restriction enzymes. The resulting pGADT7-AtSK or pGBKT7-AtSK constructs were used for yeast transformations. Protein interactions in yeast were detected using the Matchmaker Two-Hybrid System 3 (Clontech, Mountain View, CA) according to the manufacturer's instructions. Initial interactions were screened using the yeast strain AH109 co-transformed with pGADT7 and pGBKT7 constructs,

using a 1:1 ratio of vector DNA. Co-transformants were grown on dropout medium lacking adenine, histidine, leucine (Leu), and tryptophan (Trp).

For quantification of β -galactosidase expression, yeast strain Y187 was co-transformed with pGADT7-C4 and pGBKT7-AtSKs, and selected on dropout medium lacking Leu and Trp. Quantitative analysis of β -galactosidase expression was carried out on independent yeast transformants using the Galacto-Star System for yeast (Applied Biosystems Inc., Foster City, CA) following the manufacturer's protocol. Chemiluminescence was measured using a BioTek microplate reader (BioTek, VT). For each pGADT7/pGBMKT7 construct combination, three independent yeast transformants were assayed in triplicate. Relative β -galactosidase activity was calculated with respect to the C4/AtSK23 interaction.

Bimolecular fluorescence complementation

The C4 gene was amplified as above, using primers C4-EYFP 5F and C4-EYFP 3R, cloned into pBSKS+, and subsequently subcloned into the pSAT1-cEYFP-N1 vector (Citovsky et al., 2006) using *Nco*I and *Bam*HI restriction sites, which places the C4 gene (lacking the endogenous stop codon) upstream of the C-terminal portion of EYFP (cEYFP) to give pC4-cEYFP. The C4 gene was similarly cloned into pSAT1-nEYFP-N1, which fuses the C4 gene to the N-terminal portion of EYFP (nEYFP) to give pC4-nEYFP. The C4 mutant genes were similarly cloned into the pSAT1-cEYFP-N1 vector. Two exceptions were C4G2A, which used the C4G2ANco and C4-EYFP 3R primer pair, and C471Stp, which used the 71truncBam2 and C4-EYFP 5F primer pair (Table S 4-1). The AtSK genes were PCR amplified from *Arabidopsis* ecotype Sei-0 cDNA, using the primers indicated in Table 4-1, cloned into pGEM-T (Promega, Madison, WI), and subcloned with the appropriate restriction enzymes into the multiple cloning

site of either pSAT4-nEYFP-N1, for AtSK21, or pSAT4A-nEYFP-N1 for all other genes (Citovsky et al., 2006). In all cases the AtSK gene (lacking the endogenous stop codon) was placed upstream of the N-terminal portion of EYFP. The fusion protein cassettes were subcloned into the pPZP-RCS2-bar vector (Citovsky et al., 2006) using *AscI* for pSAT1-cEYFP-N1 clones and *I-SceI* for pSAT4/4A-nEYFP-N1 clones. The integrity of the genes in all plasmids was confirmed by sequencing. All pPZP-RCS2 clones were subsequently transformed into *Agrobacterium tumefaciens* strain LBA4404.

Fusion proteins were transiently expressed in *N. benthamiana* or transgenic *N. benthamiana* line CFP-H2B (Martin et al., 2009) using *A. tumefaciens* infiltration. Individual *A. tumefaciens* colonies were grown to stationary phase in LB broth with antibiotics, and then grown to an OD₆₀₀ between 0.8 and 1.0 in L-MESA medium (LB broth, 0.01 μM MES pH 5.7, 20 μM acetosyringone). Cells were harvested by centrifugation for 10 minutes at 3,500 xg, resuspended in agroinduction medium (0.01 μM MgCl₂, 0.02 μM MES pH 5.7, 100 μM acetosyringone in water) to an OD₆₀₀ between 1.0 and 1.1, and incubated at room temperature for 3-h. Cultures were combined at 1:1 ratios and infiltrated into the abaxial surface of young fully expanded *N. benthamiana* leaves using a 3 ml syringe without a needle. Infiltrated plants were incubated for 48-h at 25 °C, 16/8-h day/night cycles. Water-mounted leaf tissue sections were examined with a Zeiss LSM 510 Meta confocal microscope equipped with a Zeiss Axio Imager M1 upright microscope and lasers spanning the spectral range from 405-514 nm (AHRC, University of Georgia). Images were acquired at a resolution of 2048 x 2048 pixels, with a scan rate of 1.6 ms pixel⁻¹. All digitally captured images were formatted using Photoshop CS3. Each plasmid combination was assayed three independent times.

The C4-nEYFP or C4-cEYFP fusion proteins were PCR amplified (as above) using primer PVXC45 in combination with cEYFP_{EcoRev} or nEYFP_{EcoRev} and pC4-cEYFP or pC4-nEYFP template DNA, respectively. The resulting PCR products were digested with *Clal* and *EcoRV* restriction sites, cloned into pP2C2S, linearized with *SpeI*, and inoculated into *N. benthamiana* plants as indicated above (mutagenesis and mutant screen).

Western Analysis

Seedlings were germinated in liquid culture and grown for 7 days prior to induction with 10 μ M β -estradiol or 1 μ M brassinolide (BL) or both and harvested at 24 hours post-induction (Mills-Lujan and Deom, 2010). C4 protein was extracted as previously described (Mills-Lujan and Deom, 2010). For larger proteins, such as BES1, the same protocol was used, except tricine sample buffers were replaced with Laemmli buffer (62.5 μ M Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 2% β -mercaptoethanol, 0.01% bromophenol blue).

C4 proteins were separated, blotted and detected as previously described (Mills-Lujan and Deom, 2010). BES1 was separated in 10% Criterion Tris-glycine gels according to the manufacturer (Bio-Rad, Hercules, CA) and blotted onto nitrocellulose (Deom et al., 1987). The C4 protein was detected using affinity purified rabbit antiserum (Mills-Lujan and Deom, 2010), and the BES1 protein was detected using anti-BES1 (Yin et al., 2005) as the primary antibody. Anti-rabbit polyclonal antiserum, conjugated to alkaline phosphatase, was used as a second antibody for visualization for C4 (ProtoBlot II AP System, Promega, Madison, WI). Anti-rabbit polyclonal antiserum, conjugated to horseradish peroxidase, was used as a second antibody for visualization with SuperSignal Pico West (Thermo Fisher Scientific, Rockford, IL), followed by exposure to HyperfilmTM ECL X-ray film (Amersham Biosciences, Piscataway, NJ).

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Conflict of Interest The authors declare that they have no conflict of interest.

Figures.

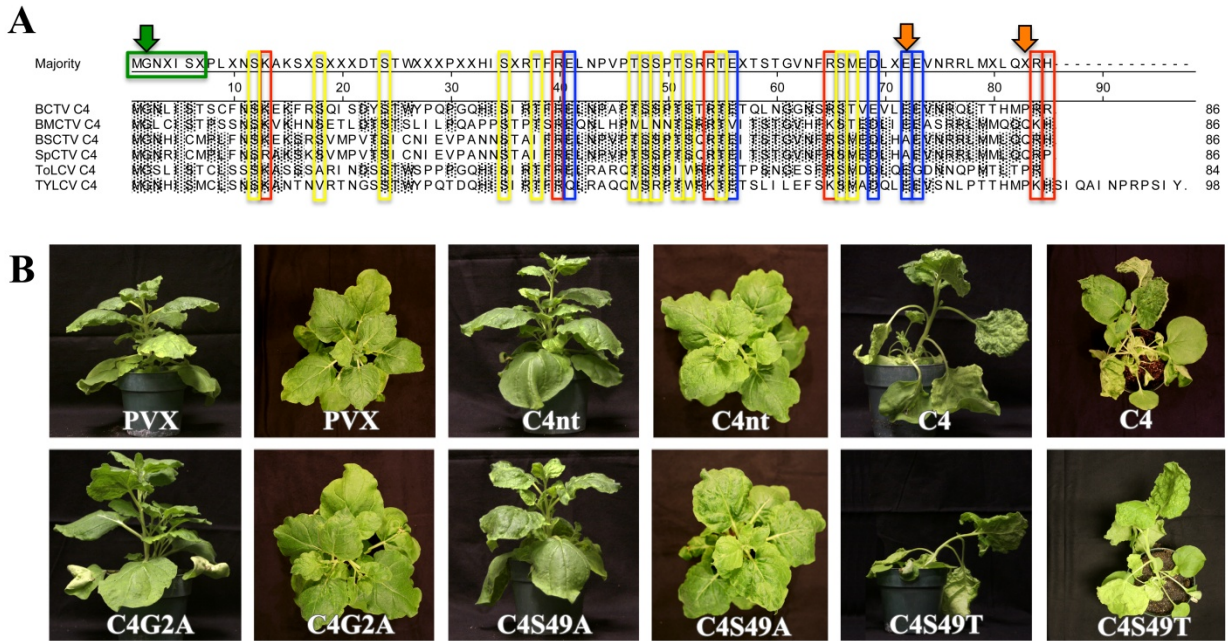


Fig. 4.1. Screening of C4 mutants in *Nicotiana benthamiana*. **(A)** Alignment of C4 proteins from *Beet curly top virus* (BCTV), *Beet mild curly top virus* (BMCTV), *Beet severe curly top virus* (BSCTV), *Spinach curly top virus* (SpCTV), *Tomato yellow leaf curl virus* (TYLCV), and *Tomato leaf curl virus* (ToLCV). Mutations of positively charged residues (Lys and Arg) to Ala boxed in red, mutations to negatively charged residues (Asp and Glu) to Ala boxed in blue, mutations of Ser and Thr residues to Ala boxed in yellow, and mutation of Gly in the N-myristoylation motif (green box) to Ala indicated with a green arrow. A, alanine; D, aspartic acid; E, glutamic acid; K, lysine; R, arginine; S, serine; T, threonine. **(B)** C4 mutants were inoculated into *N. benthamiana* plants using transcripts generated from a PVX-based vector. Wild type C4 was used as a positive control. A non-translatable version of C4 (C4nt) and empty PVX vector were used as negative controls. Plants shown at 14 days post inoculation. PVX, potato virus X.

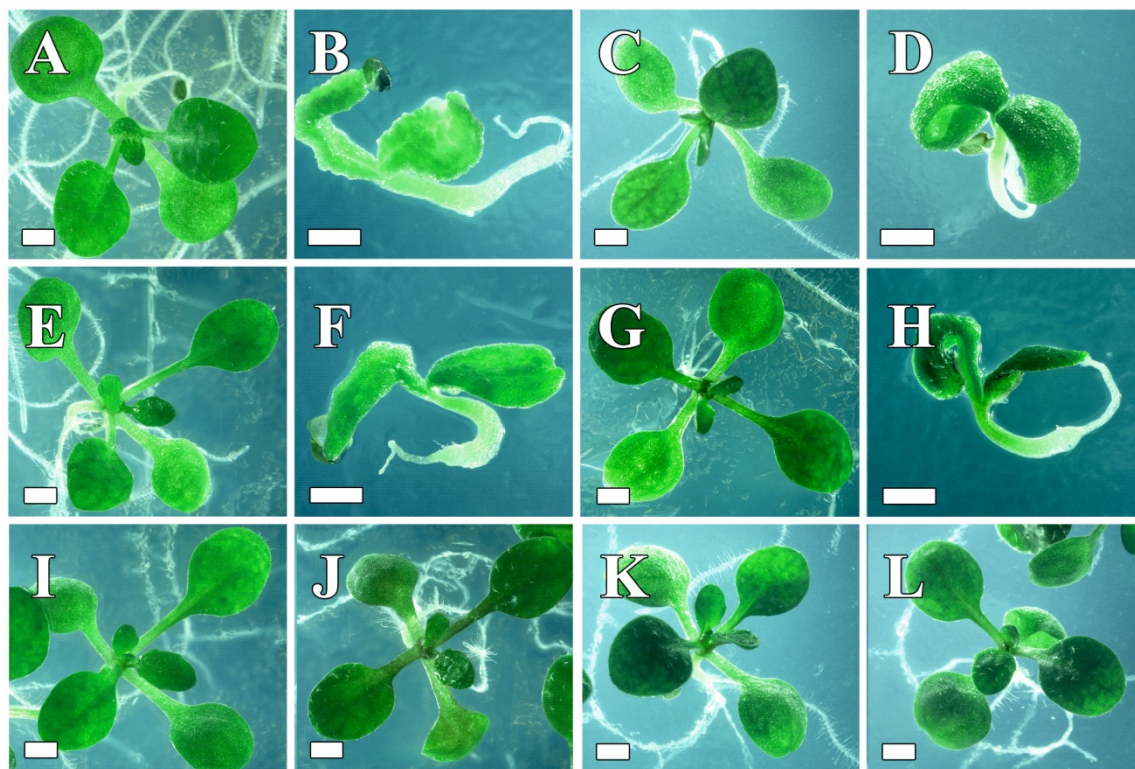


Fig. 4.2. Threonine can substitute for Serine at residue 49 in transgenic *Arabidopsis* seedlings. Transgenic *Arabidopsis* seedlings were germinated on solid media (A, C, E, G, I, and K) or solid induction media (B, D, F, H, J, and L). IPC4-28 (A and B), IPC4-18 (C and D), IPC4S49T-2 (E and F), IPC4S49T-6 (G and H), IPC4S49A-2 (I and J), and IPC4nt-12 (K and L). Scale bar = 1000 μ m

Fig. 4.3. Putative phosphorylation site on C4 is required for interaction with subgroup 2 AtSKs. Comparison of the binding between C4 and C4 mutants to AtSKs protein kinases. **(A)** Interaction between C4 and the ten different AtSK family members. Interaction between C4 and Lamin was used as a negative control. **(B)** Comparison of the interaction between C4 and sg2 AtSK kinase dead (KD) and gain-of-function (GF) mutants. The interaction between C4 and wild type sg2 AtSKs is shown as a positive control, and C4/Lamin as a negative control. **(C)** Comparison of the interaction between Ser49 mutants of C4 (C4S49A, C4S49T, C4S49E) and sg2 AtSKs, and wild type C4 and sg2 AtSKs. The N-myristoylation mutant (C4G2A) was used a control. β -galactosidase activity was quantified using triplicate samples from three individual yeast colonies, and is expressed relative to the C4/AtSK23 interaction.

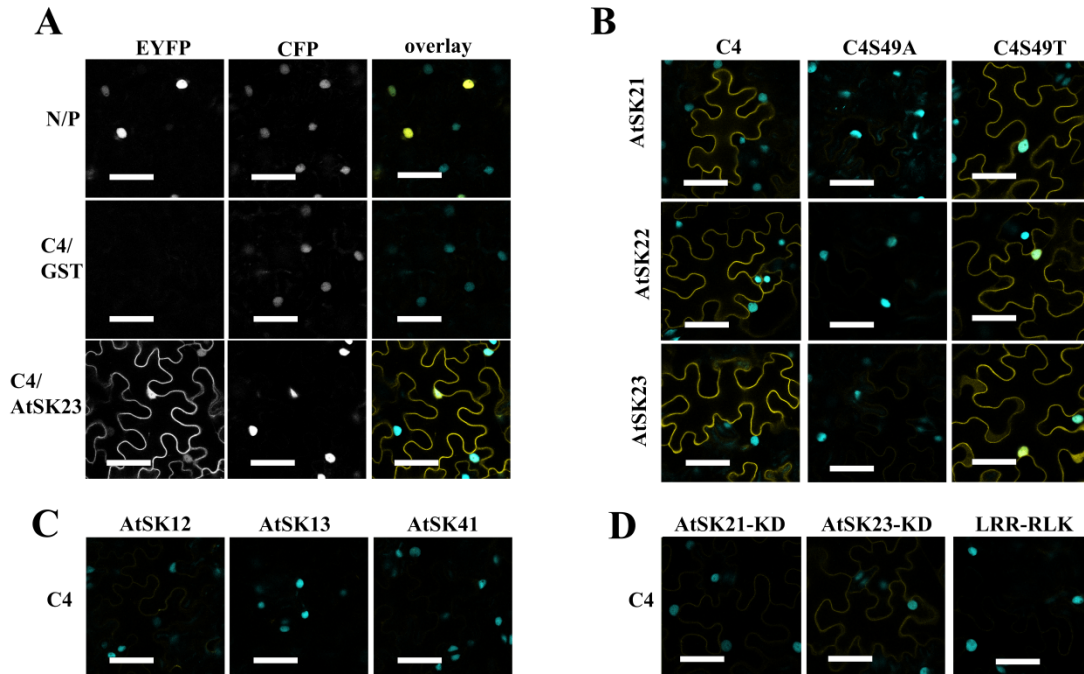


Fig. 4.4. C4 interacts with sg2 AtSKs *in planta* using bimolecular fluorescence complementation (BiFC). Confocal micrographs showing differential interactions between C4 and C4 mutants with AtSKs and sg2 AtSK mutants co-agroinfiltrated into *N. benthamiana* CFP-H2B plants. Reconstituted EYFP, marker line CFP and overlay of both fluorescent signals are shown in (A). Only the overlay of EYFP and CFP signals are shown in (B-D). (A) EYFP signal (yellow) from SYN-V-N and SYN-V-P positive control interaction co-localizes with fluorescence from CFP-H2B (blue) in the nucleus, as seen in the overlay (upper row). No EYFP signal was detected in the GST/C4 negative control (middle row). AtSK23 and C4 interact at the plasma membrane and in the nucleus (yellow; bottom row). (B) C4 and C4S49T interact with AtSK21, AtSK22 and AtSK23 at the plasma membrane and in the nucleus (yellow, first and third column). No interaction was detected between C4S49A and AtSK21, AtSK22 and AtSK23, resulting in only CFP-H2B fluorescence being visible in the nucleus (second column). (C) C4 does not interact with AtSK12, AtSK13, and AtSK41, as only the CFP signal is visible in the overlay. (D) C4 interacts very weakly with AtSK21-KD and AtSK23-KD mutants, and does not interact with LRR-RLK. Scale bar = 50 μ M

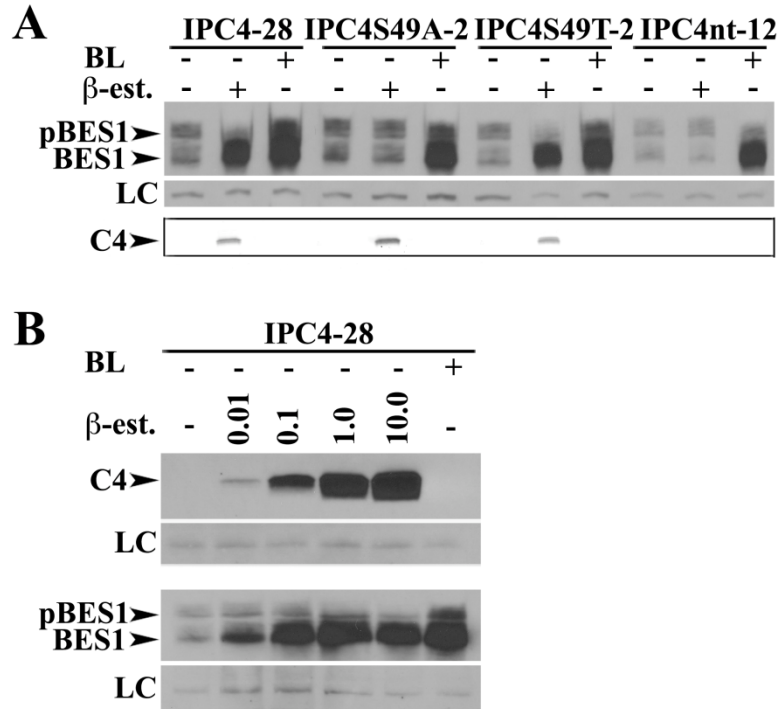


Fig. 4.5. C4S49A mutation abolishes effect of C4 on BR signaling pathway. IPC4-28, IPC4S49A and IPC4S49T seedlings were germinated for 7 days prior to induction, collected 24 hours post-induction, and assayed by Western blot analysis. **(A)** Phosphorylation status of BES1 in non-induced, β-estradiol or BL induced IPC4-28, IPC4S49A-2, IPC4S49T-2, or negative control IPC4nt-12 line (upper panel). The corresponding C4 expression levels are shown in the lower panel. **(B)** IPC4-28 seedlings induced with increasing concentrations of β-estradiol (0.01, 0.1, 1.0, 10.0 μM), BL only or non-induced control. Increasing levels of C4 shown in upper panel, with corresponding shift in BES1 phosphorylation status shown in lower panel. Non-specific loading control band shown below each respective western blot. BL, brassinolide; β-est., β-estradiol; LC, loading control; pBES1, hyperphosphorylated BES1.



Figure S 4.1 Screening of C4 mutants in *Nicotiana benthamiana*. **(A)** C4 mutants were inoculated into *N. benthamiana* plants using a PVX-based vector. Wild type C4 was used as a positive control. A non-translatable version of C4 (C4nt) and empty PVX vector were used as negative controls. Plants shown at 14 days post inoculation. **(B)** C4 tagged with the N-terminal portion of EYFP (nEYFP) or the C-terminal portion of EYFP (cEYFP) was inoculated into *N. benthamiana* using a PVX-based vector. Plants inoculated with C4nt negative control, C4 positive control and C4-fusion protein shown in lateral view, C4-fusion proteins shown from top view. Third systemic leaf collected from control and C4-fusion protein inoculated plants are also shown. PVX, potato virus X.

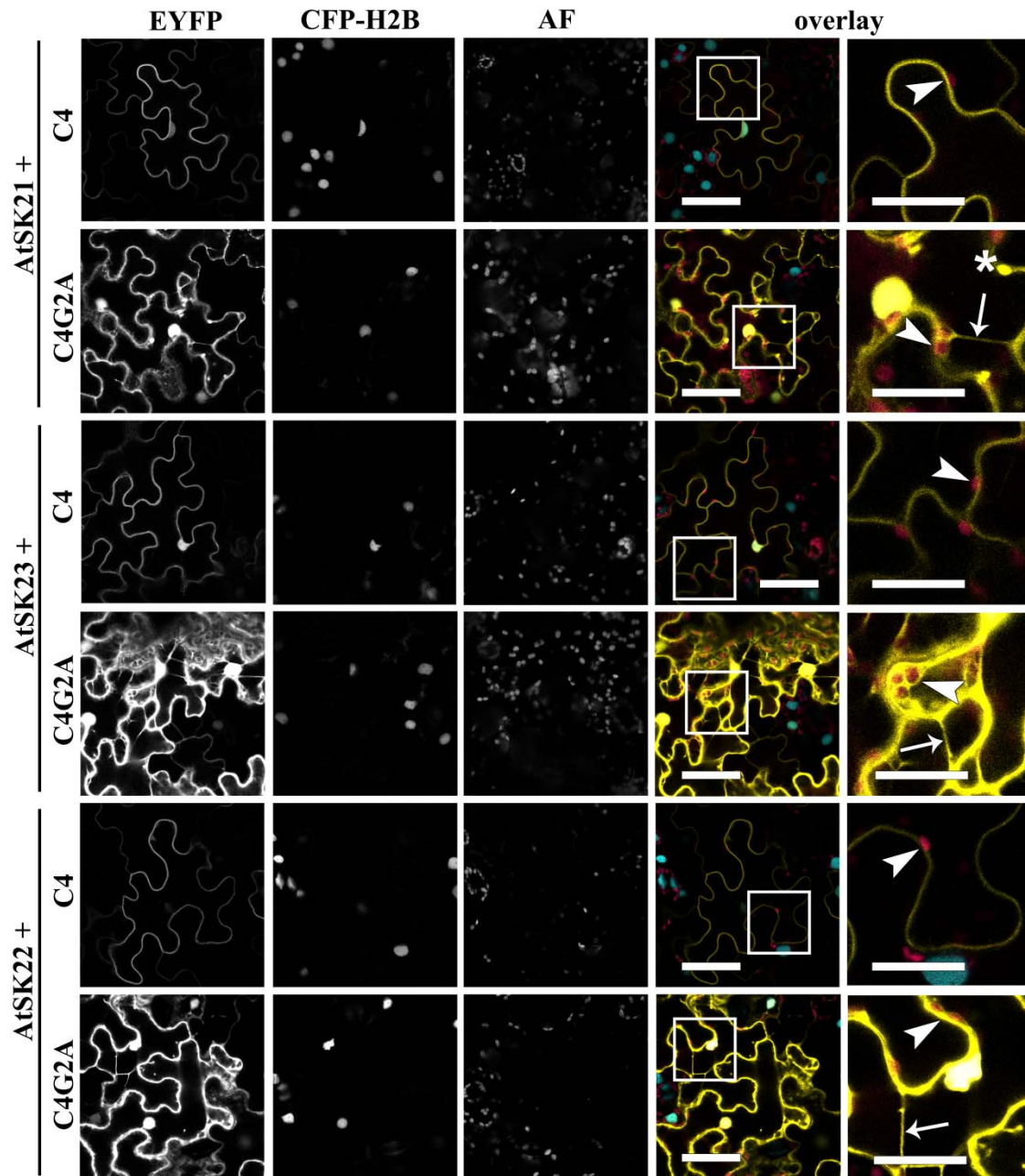


Figure S 4.2 N-myristoylation motif on C4 is required for C4/sg2 AtSK interaction at the plasma membrane. Confocal micrographs showing differential interactions between cEYFP-tagged C4 and C4G2A mutant with nEYFP-tagged subgroup 2 AtSK co-infiltrated into *N. benthamiana* CFP-H2B lines. Reconstituted EYFP (first column), marker line CFP (second column), chloroplast autofluorescence (AF; third column) and overlay of both fluorescent signals (fourth) and insert from overlay (fifth column) are shown. Arrowhead, chloroplasts; arrow, cytoplasmic strands; asterisk, cytoplasmic granule. Scale bar = 50 μ M.

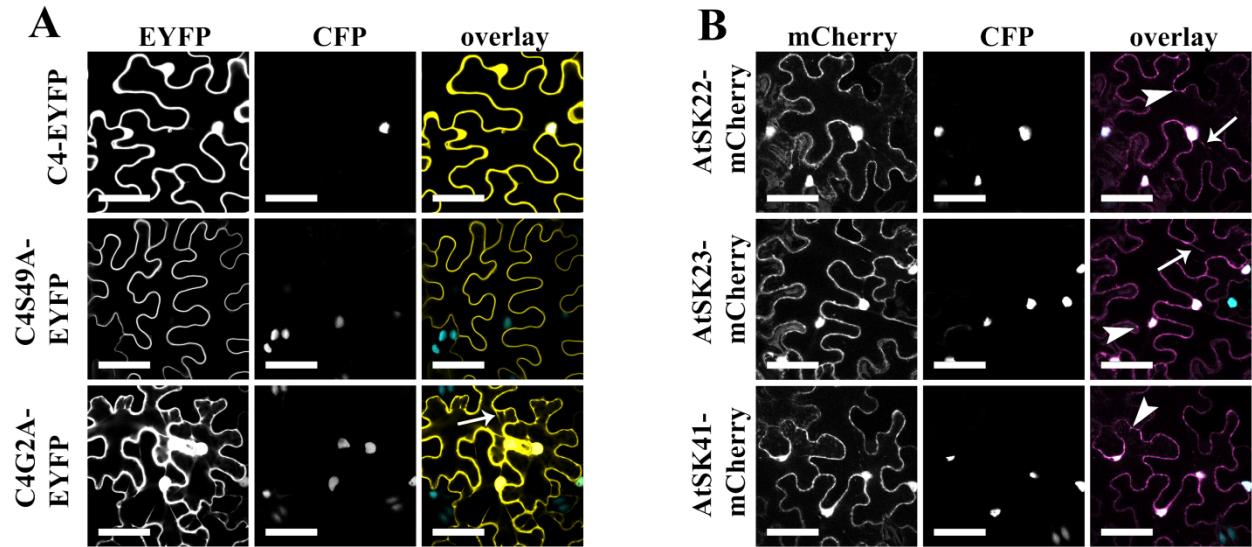


Figure S 4.3 C4S49A localizes to the plasma membrane. C4, C4S49A, C4G2A fused to intact EYFP (A) and AtSK22, AtSK23, and AtSK41 fused to mCherry (B) were agro-infiltrated into *N. benthamiana* CFP-H2B lines. (A) C4-EYFP and C4S49A-EYFP localize to the plasma membrane. C4-EYFP also localized to the nucleus, as seen in the overlay with nuclear CFP reference signal. C4G2A localizes to the cytoplasm and the nucleus. (B) AtSK22-, AtSK23- and AtSK41-mCherry fusion proteins localize to the cytoplasm, evidenced by cytoplasmic strands (arrow) and cytoplasmic granules (arrowhead). The mCherry signal also co-localized with the CFP reference nuclear signal (CFP and overlay). Arrowhead, cytoplasmic granule; arrow, cytoplasmic strands. Scale bar = 50 μ M.

Tables

Table 4-1 Sequence of PCR primers used for cloning genes

Primer name	Primer Sequence ¹	RE ²	Target gene
PVXC45	CCGATCGATAACAATGGGCAACCTCATCTCCACGTCC	<i>Clal</i>	BCTV C4
PVXC43	CCGGATATCTTAACGCCTTGGCATATGAGTCG	<i>EcoRV</i>	
C4NTE103	CGGGATATCACGCCTTGGCATATGAGTCG	<i>EcoRV</i>	BCTV C4
C4G2ApER5	CGGCTCGAGAACAATGGCCAACCTCATCTCC	<i>XhoI</i>	C4G2A
cEYFP EcoRev	CCGGATATCTCACTTGTACAGCTCGTCCATGC	<i>EcoRV</i>	C4-cEYFP
nEYFP EcoRev	CCGGATATCTCAGTCCTCGATGTTGTGGCGGA	<i>EcoRV</i>	C4-nEYFP
C4-EYFP 5F	CCGCCATGGGCAACCTCATCTCCACG	<i>NcoI</i>	BCTV C4
C4-EYFP 3R	CCGGGATCCCACGCCTTGGCATATGAGTCGT	<i>BamHI</i>	
BIN2-EYFP 5F	CCGCCATGGCTGATGATAAGGAGATG	<i>NcoI</i>	AtSK21
BIN2-EYFP 3R	CCGGGATCCCAGTTCAGATTGATTCAAGAA	<i>BamHI</i>	(At4g18710) ³
SK22longEcoF	CCGGAATTCATGGCCTCATTACCATTGGGG	<i>EcoRI</i>	AtSK22
SK22BamHI3R	CCGGGATCCTTAACCTGTTTTGTAATCCTGT	<i>BamHI</i>	(At1g06390) ³
SK23-EYFP 5F	CCGAATTCATGACTTCGATACCATTGGGG	<i>EcoRI</i>	AtSK23
SK23BamHI3R	CCGGGATCCCTAGGGTCCAGCTTGAAATGG	<i>BamHI</i>	(At2g30980) ³
SK41-EY 5F	CCGCTCGAGATGGCATCCTCTGGACTGGGA	<i>EcoRI</i>	AtSK41
SK41BamHI3R	CCGGGATCCTTACGAATGCAAAGCCATGAA	<i>BamHI</i>	(At1g09840) ³
At3g24240 5F	CCGGAATTCATGTCTCTTATTCTTAATC	<i>EcoRI</i>	LRR-RLK
At3g24240 3R	CCGGGATCCCCTCGATTGAAGAAGAAGA	<i>BamHI</i>	(At3g24240) ³
AtSK11 H3 5F	CCGAAGCTTATGGCGTCAGTGGGTATAGCT	<i>HindIII</i>	AtSK11
AtSK11 B 3R	CCGGGATCCCCAAACCGAGCCAAGGACACT	<i>BamHI</i>	(At5g26751) ³
AtSK12 H3 5F	CCGAAGCTTATGGCCTCGGTGGGCATAGA	<i>HindIII</i>	AtSK12
AtSK12 B 3R	CCGGGATCCCCAAACTGAGCCACGGACATT	<i>BamHI</i>	(At3g05840) ³
AtSK13 N 5F	CCGCCATGGCTTCTGTGGGAACATTA	<i>NcoI</i>	AtSK13
AtSK13 B 3R	CCGGGATCCCAGAGCGAGGAAGGAACATT	<i>BamHI</i>	(At5g14640) ³
AtSK31 N 5F	CCGGAATTCATGAATGTGGTGCGGAGATTA	<i>NcoI</i>	AtSK31
AtSK31 B 3R	CCGGGATCCCTTTCTTGCATGCTCAGGTA	<i>BamHI</i>	(At3g61140) ³
AtSK32 N 5F	CCGAGATCTATGAACGTGATGCGTCGTCT	<i>NcoI</i>	AtSK32
AtSK32 ERI 3R	CCGGAATTCAGAGCTACTTCCCGTTCCCT	<i>EcoRI</i>	(At4g00720) ³
SK42 Nco5F	CCGCCATGGAATTCATCTGGGAAATGGA	<i>NcoI</i>	AtSK42
AtSK42 B 3R	CCGGGATCCCCGAATGCAAAGCCATGAAGA	<i>BamHI</i>	(At1g57870) ³
BaitC45PR	CGCCATGGGCAACCTCATCTCCACG	<i>NcoI</i>	BCTV C4
BDC43PR	CGGGATCCTTAACGCCTTGGCATATGAGTCG	<i>BamHI</i>	
C4G2A5Nco	CGCCATGGCCAACCTCATCTCCACG	<i>NcoI</i>	C4G2A
72StpBamHI	CCGGGATCCTTAACGCCTTGGCATATGAGT	<i>BamHI</i>	C4-71Stp
SK22BamHI3R	CCGGGATCCTTAACCTGTTTTGTAATCCTGT	<i>BamHI</i>	AtSK22 ⁴
SK23BamHI3R	CCGGGATCCCTAGGGTCCAGCTTGAAATGG	<i>BamHI</i>	AtSK23 ⁴
SK41BamHI3R	CCGGGATCCTTACGAATGCAAAGCCATGAA	<i>BamHI</i>	AtSK41 ⁴
LRRBamHI 3R	CCGGGATCCTCACTCGATTGAAGAAGAAGA	<i>BamHI</i>	LRR-RLK ⁴
AtSK11 Nde 5F	CCGCATATGGCGTCAGTGGGTATAGCT	<i>NdeI</i>	AtSK11 ⁵
SK11 B STP	CCGGGATCCTCACAAACCGAGCCAAGGACA	<i>BamHI</i>	
AtSK12 Nde 5F	CCGCATATGGCCTCGGTGGGCATAGA	<i>NdeI</i>	AtSK12 ⁵
SK12 B STP	CCGGGATCCTCACAAACTGAGCCACGGACA	<i>BamHI</i>	
SK13 B STP	CCGGGATCCTTAGAGAGCGAGGAAGGAACA	<i>BamHI</i>	AtSK13 ⁴
SK31 B STP	CCGGGATCCTCATTCTTGCATGCTCAGG	<i>BamHI</i>	AtSK31 ⁴
AtSK32 ERI 5F	CCGGAATTCATGAACGTGATGCGTCGTCT	<i>EcoRI</i>	AtSK32 ⁵
AtSK32 Sal 3R	CCGGTTCGACCTAAGAGCTACTTCCCGTTCC	<i>SalI</i>	
AtSK42 N 5F	CCGCCATGGCATCCTCTGGACTGGGA	<i>NcoI</i>	AtSK42 ⁵

SK42 B STP	CCGGGATCCTTACGAGTGTAATGCCATGAA	<i>BamHI</i>	
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¹ Restriction site is underlined.

² RE = restriction enzyme used for cloning.

³ Primer pair for cloning gene into pSAT vectors for BiFC.

⁴ For cloning gene into pGBKT7 for yeast two-hybrid. This 3' primer used in combination with 5' primer for pSAT vector cloning.

⁵ Primer pair for cloning gene into pGBKT7 for yeast two-hybrid.

Table 4-2 Summary of results for C4 mutant screen. C4 mutants were inoculated into *Nicotiana benthamiana* using a PVX-based vector. Phenotype was scored as C4-like or PVX-like at 14 days post inoculation.

C4 mutant ¹	Observed phenotype	C4 mutant ¹	Observed phenotype
Non-sense mutation		Serine/Threonine mutants	
C4 72Stp	PVX-like	C4S12A or E	C4-like
C4 83Stp	C4-like	C4S18A or E or T	C4-like ³
		C4S24A or E	C4-like
N-myristoylation mutant		C4S35A or E	C4-like
C4G2A	PVX-like	C4S48A	C4-like
		C4S48E	PVX-like
Acidic amino acid mutations		C4S48T	C4-like
C4E41A	C4-like	C4S49A	PVX-like
C4E56A	C4-like	C4S49E	PVX-like
C4E69A	C4-like	C4S49T	C4-like
C4E72A	C4-like	C4S52A or E or T	C4-like
C4E73A	C4-like	C4S66A or E	C4-like
		C4T38E	C4-like
Basic amino acid mutations		C4T47A or E	C4-like
C4K13A	C4-like ²	C4T51A or E	C4-like
C4R40A	C4-like	C4T55A or E	C4-like ⁴
C4R54A	C4-like	C4T67A or E	C4-like ⁵
C4R65A	C4-like		
C4R65E	C4-like	Double mutants	
C4R84A	C4-like	C4R40AE41A	C4-like
C4R85A	C4-like	C4E72AE73A	C4-like
		C4S52AR54A	C4-like

¹ Mutant designation, point mutants: C4(Original amino acid)(residue position)(New amino acid)

Mutant designation, non-sense mutants: C4(position of new stop codon)Stp

² Milder C4-like, no stem curling and less discoloration along leaf veins

³ Very mild phenotype for C4S18E, leaf surface is smooth, no stem curling or petiole rolling, only mild leaf curling on older leaves.

⁴ Symptom onset delayed 2 to 3 days for C4T55E, symptoms C4-like

⁵ Slightly milder C4-like, less stem curling and petiole rolling

Table S 4-1 Primers used for C4 and AtSK mutagenesis

Primer name	Primer sequence	Mutant ¹
C4K13F	TGCTTCAACTCGGCGGAAAAGTTCAGATC	C4K13A
C4K13R	TCTGAAC TTTCCGCCGAGTTGAAGCAGG	
C4R40F	CCGAACATTCGCGGAGCTAAATCCAGCTCCG	C4R40A
C4R40R	GCTGGATTTAGCTCCGCGAATGTTCCGATG	
C4E41F	CGAACATTCAGGGCGCTAAATCCAGCTCC	C4E41A
C4E41R	TGGATTTAGCGCCCTGAATGTTCCGATGG	
C4RE40F	TCCATCCGAACATTCGCGGGCGCTAAATCCAGCTCCG	C4R40AE41A (double mut.)
C4RE40R	AGCTGGATTTAGCGCCGCGAATGTTCCGATGGAAATGTG	
C4R65F	GGGGGAATTCCGCATCGACGGTAGAAG	C4R65A
C4R65R	CTACCGTCGATGCGGAATCCCCCATTC	
C4E69F	TCGACGGTAGCAGTGCTAGAGGAGG	C4E69A
C4E69R	CTCTAGCACTGCTACCGTCGATCTGGA	
C4R54F	CCTACGTCGACAGCGACGGAGACAC	C4R54A
C4R54R	GTGTCTCCGTCGCTGTCGACGTAGGAC	
C4E56F	ACAAGGACGGCGACACAATTGAATGGGG	C4E56A
C4E56R	CAATTGTGTCGCCGTCCTTGTGACG	
C4E72F	GAAGTGCTAGCGGAGGTCAACAGAC	C4E72A
C4E72R	GTTGACCTCCGCTAGCACTTCTACCG	
C4E73F	GTGCTAGAGGCGGTCAACAGACAGC	C4E73A
C4E73R	GTCTGTTGACCGCCTCTAGCACTTCTAC	
C4EE72-3F	GTAGAAGTGCTAGCGGCGGTCAACAGACAGC	C4E72A,E73A
C4EE72-3R	GTCTGTTGACCGCCGCTAGCACTTCTACCG	
C4R84F	TCATATGCCAGCGCGTTAAGATATCGAATTCC	C4R84A
C4R84R	TATCTTAACGCGCTGGCATATGAGTCGTTAGC	
C4R85F	TATGCCAAGGGCTTAAGATATCGAATTCCTGC	C4R85A
C4R85R	TTCGATATCTTAAGCCCTTGGCATATGAGTCG	
C4RR84-5F	CGACTCATATGCCAGCGGCTTAAGATATCGAATTCCTGC	C4R84A,R85A
C4RR84-5R	GAATTCGATATCTTAAGCCGCTGGCATATGAGTCGTTAGC	
C4S52F	TCAAGTCCTACGCGCACAAGGACGG	C4S52A
C4S52R	GTCTTGTGCGCGTAGGACTTGACG	
C4S52TF	TCAAGTCCTACGACGACAAGGACGG	C4S52T
C4S52TR	GGTCCTTGTGTCGTCGTAGGACTTGACG	
C4S52R54F	ACGTCAAGTCCTACGGCGACAGCGACGGAGACACAA	C4S52A,R54A
C4S52R54R	GTGTCTCCGTCGCTGTCGCCGTCGACTTGACGTCG	
S66AFor	GGGAATTCAGAGCGACGGTAGAAGTGC	C4S66A
S66ARev	CTTCTACCGTCGCTCTGGAATTC CCCCC	
T67AFor	GAATTCAGATCGGCGGTAGAAGTGCTAG	C4T67A
T67ARev	GCACTTCTACCGCCGATCTGGAATTCC	
R65EFor	GGGGGAATTCCGAATCGACGGTAGAAG	C4R65E
R65ERev	CTACCGTCGATTCGGAATTC CCCCCATTC	
C472StpFOR	GTAGAAGTGCTATAGGAGGTCAACAGAC	C472Stp
C472StpREV	TCTGTTGACCTCCTATAGCACTTCTACCG	
C4G2A FOR	ATCGATAACAATGGCCAACCTCATCTCC	C4G2A
C4G2A REV	GGAGATGAGGTTGGCCATTGTTATCGATACC	
C4S12A FOR	TCCTGCTTCAACGCGAAGGAAAAGTTCAG	C4S12A
C4S12A REV	GAAC TTTTCTTCGCGTTGAAGCAGGACG	
C4S24A FOR	ATATCAGATTATGCGACCTGGTATCCC	C4S24A
C4S24A REV	GGATACCAGGTGCGATAATCTGATATTTG	
C4S35A FOR	CAGCACATTGCCATCCGAACATTCAG	C4S35A
C4S35A REV	GAATGTTCCGATGGCAATGTGCTGACC	

C4S48A FOR	GCTCCGACGGCAAGTCCTACGTCG	C4S48A
C4S48A REV	CGTAGGACTTGCCGTCGGAGCTGG	
C4S49A FOR	GCTCCGACGTCAGCTCCTACGTCGACAAGG	C4S49A
C4S49A REV	TGTCGACGTAGGAGCTGACGTCGGAGCTGG	
C4T47A FOR	CCAGCTCCGGCGTCAAGTCCTACG	C4T47A
C4T47A REV	CGTAGGACTTGACGCCGGAGCTGG	
C4T51A FOR	ACGTCAAGTCCTGCGTCGACAAGGAC	C4T51A
C4T51A REV	CTTGTCGACGCAGGACTTGACGTCGG	
C4T55A FOR	GTCGACAAGGGCGGAGACACAATTG	C4T55A
C4T55A REV	CAATTGTGTCTCCGCCCTTGTGACG	
C483Stp For	CGACTCATATGCCATGACGTAAAGATATCGAATTC	C483Stp
C483Stp Rev	CGATATCTTAAACGTCATGGCATATGAGTCGTTAG	
C4S12E For	TCCTGCTTCAACGAGAAGGAAAAGTTCAGATCAC	C4S12E
C4S12E Rev	CTGAACTTTTCCTTCTCGTTGAAGCAGGACGTGG	
C4S18E For	GAAAAGTTCAGAGAACAATATCAGATTATTCGACC	C4S18E
C4S18E Rev	ATAATCTGATATTTGTTCTCTGAACTTTTCCTTCGAG	
C4S24E For	TCACAAATATCAGATTATGAGACCTGGTATCCCC	C4S24E
C4S24E Rev	GGGGATACCAGGTCTCATAATCTGATATTTGTGATC	
C4S35E For	GGTCAGCACATTGAGATCCGAACATTCAGGGAG	C4S35E
C4S35E Rev	GAATGTTCCGATCTCAATGTGCTGACCTGGTTG	
C4S38E For	CACATTTCCATCCGAGAATTCAGGGAGCTAAATCC	C4S38E
C4S38E Rev	TAGTCCCTGAATTCTCGGATGGAAATGTGC	
C4S48E For	CCAGCTCCGACGGAAAGTCCTACGTCGACA	C4S48E
C4S48E Rev	CGACGTAGGACTTTCCGTCGGAGCTGG	
C4S49E For	GCTCCGACGTCAGAACCTACGTCGACAAGG	C4S49E
C4S49E Rev	TGTCGACGTAGGTTCTGACGTCGGAGCTGG	
C4S52E For	ACGTCAAGTCCTACGGAGACAAGGACGGAGAC	C4S52E
C4S52E Rev	CGTCCTTGTCTCCGTAGGACTTGACGTCGG	
C4S66E For	GGGAATTCCAGAGAGACGGTAGAAGTGCTAGAG	C4S66E
C4S66E Rev	GCACTTCTACCGTCTCTCTGGAATTCCTCCCC	
C4T47E For	AATCCAGCTCCGGAGTCAAGTCCTACGTCG	C4T47E
C4T47E Rev	CGTAGGACTTGACTCCGGAGCTGGATTTAGC	
C4T51E For	ACGTCAAGTCCTGAGTCGACAAGGACGGAG	C4T51E
C4T51E Rev	CGTCCTTGTGCGACTCAGGACTTGACGTCGG	
C4T55E For	ACGTCGACAAGGGAGGAGACACAATTGATTGGG	C5T55E
C4T55E Rev	CAATTGTGTCTCCTCCCTTGTGACGCTAGG	
C4T67E For	GAATTCAGATCGGAGGTAGAAGTGCTAGAG	C4T67E
C4T67E Rev	CTAGCACTTCTACCTCCGATCTGGAATTTCC	
C4E18T FOR	GAAAAGTTCAGAACACAAATATCAGATTATTCGACC	C4S18T
C4E18T REV	ATAATCTGATATTTGTGTTCTGAACTTTTCCTTCGAG	
C4A48T FOR	GCTCCGACGACAAGTCCTACGTCG	C4S48T
C4A48T REV	CGTAGGACTAGTCGTCGGAGCTGG	
C4A49T FOR	TCCGACGTCAACTCCTACGTCGACAAG	C4S49T
C4A49T REV	TGTCGACGTAGGAGTTGACGTCGGAGC	
C4E49T FOR	GCTCCGACGTC AACACCTACGTCGACAAGG	C4S49T
C4E49T REV	TGTCGACGTAGGTGTTGACGTCGGAGCTGG	
C4S18A FOR	GAAAAGTTCAGAGCACAAATATCAGATTATTCG	C4S18A
C4S18A REV	ATCTGATATTTGTGCTCTGAACTTTTCCTTCGA	
SK21K69R F	GAA ACC GTG GC G ATA CGG AAG GTT TTG CAA GAT	AtSK21K69R
SK21K69R R	ATC TTG CAA AAC CTT CCG TAT CGC CAC GGT TTC	
SK21E263K F	GGT ACA CCA ACT CGA AAA GAA ATC CGT TGT ATG	AtSK21E263K
SK21E263K R	CAT ACA ACG GAT TTC TTT TCG AGT TGG TGT ACC	
SK22K99R F	GAA TCA GTA GCC ATT CGA AAG GTC TTG CAA GAT	AtSK22K99R

SK22K99R R	ATC TTG CAA GAC CTT TCG AAT GGC TAC TGA TTC	
SK22E291K F	GGT ACT CCA ACT CGT AAA GAA ATT CGA TGT ATG	AtK22E293K
SK22E291K R	CAT ACA TCG AAT TTC TTT ACG AGT TGG AGT ACC	
SK23K101R F	GAA TCA GTA GCC ATT CGG AAG GTT TTG CAA GAT	AtSK23K101R
SK23K101R R	ATC TTG CAA AAC CTT CCG AAT GGC TAC TGA TTC	
SK23E293K F	GGT ACT CCA ACT CGC AAA GAA ATC CGG TGC ATG	AtSK23E295K
SK23E293K R	CAT GCA CCG GAT TTC TTT GCG AGT TGG AGT ACC	

¹ Mutant designation: GeneName(Original amino acid)(residue position)(New amino acid)

CHAPTER 5

BEET MILD CURLY TOP VIRUS C4, HOW DOES IT DIFFER FROM BEET CURLY TOP VIRUS C4?³

³ Mills-Lujan, Katherine and C. Michael Deom. To be submitted to *Virology*.

Introduction

Curly top viruses belong to the genus *Curtovirus* (family *Geminiviridae*) characterized by single stranded DNA and transmitted by leafhoppers (*Circulifer tenellus* Baker) (Gutierrez, 1999; Hanley-Bowdoin et al., 1999). The curtovirus genome is monopartite, ~2.9kb, and encodes seven open reading frames (ORFs). Three are highly conserved genes in the virion-sense, V1, V2, and V3, and four more divergent genes on the complimentary-sense, C1, C2, C3, and C4 (Stanley et al., 1986; Stenger, 1994; Baliji et al., 2004). The V1 ORF encodes the multifunctional coat protein (CP), which is important for virion formation, systemic infection and insect transmission. The V3 ORF is involved in systemic movement, and the V2 gene appears to regulate progeny DNA levels (Briddon et al., 1989; Hormuzdi and Bisaro, 1993; Soto et al., 2005). The C1 and C3 ORFs are homologous to the AC1 and AC3 genes in other geminiviruses and correspond to the essential Rep protein that is involved in viral DNA replication, and the dispensable REn, which enhances viral DNA accumulation (Briddon et al., 1989; Hormuzdi and Bisaro, 1995). The C2 ORF does not share the transcription activation activity of the AC2 homologue, but both AC2 and C2 have been shown to modify host plant defenses by inactivating SNF1 kinase (Hao et al., 2003). The C4 ORF is a pathogenicity determinant (Stanley and Latham, 1992).

Curly top disease (CTD) of sugarbeets is caused by three closely related viruses, *Beet curly top virus* (BCTV), *Beet mild curly top virus* (BMCTV) and *Beet severe curly top virus* (BSCTV) (Stenger et al., 1990; Stenger, 1998), which can cause severe losses on bean, cucurbits, pepper, and tomato (Bennett, 1971; Strausbaugh et al., 2008). Curly top viruses and the leafhopper vectors that transmit them can survive or overwinter on hundreds of plant species (Creamer et al., 1996). Originally, these three viruses were considered to be different strains of

BCTV, but due to differences in their symptom severity and inter-species replication specificity they were divided into the three different species recognized today (Lee et al., 1994; Stenger, 1998). However, how the symptom severity relates to virus replication, host cell modification, and transmission has not been characterized. The C4 gene of BCTV may function through disrupting the brassinosteroid (BR) signaling pathway and possibly other hormone pathways (Mills-Lujan and Deom, 2010; and Chapter 4), whereas the C4 gene of BSCTV affects the function of ubiquitin E3 ligase RKP (Lai et al., 2009). Due to the divergence in the C4 protein sequence between BCTV, BMCTV, and BSCTV (Fig. 4.1), the role of curtovirus C4 gene in symptom development was investigated by comparing the function of the BCTV C4 (referred to hereon as C4) and BMCTV C4 (referred to hereon as BMC4) genes. The results presented in this chapter are preliminary. Implications for the results shown herein, and the future direction of this project are discussed.

Materials and Methods

Mutagenesis and mutant screen

In order to carry out site-directed mutagenesis, the BMC4 gene was amplified by PCR (Mills-Lujan and Deom, 2010), using primers BMC4-5Cla and BMC4-3EcoRV. The PCR product was cloned into pBSKS+ (Stratagene, La Jolla, CA) to give pBSKS-BMC4. Site-directed mutagenesis of the C4 gene was carried out using QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with pBSKS-BMC4 as a template and the primers in (Table 5-1), following the manufacturer's instructions. BMC4 mutants (indicated in Table 5-1) were excised using *ClaI* and *EcoRV* and subcloned into the PVX-based expression vector

pP2C2S (Chapman et al., 1992) to give 3 different pP2C2S-BMC4 mutant constructs. The integrity of the BMC4 and BMC4 mutant genes in all plasmids was confirmed by sequencing.

Generation of Transgenic *Arabidopsis* Plant Lines

The BMC4 gene was PCR-amplified, using primers BMC45 and BMC43. The PCR products were cloned into pER10 as previously described (Mills-Lujan and Deom, 2010), to give pERBMC4. Transforming *Arabidopsis thaliana* ecotype Sei-0 and screened for single-copy insertion homozygotes were as previously described (Clough and Bent, 1998; Mills-Lujan and Deom, 2010).

Yeast two-hybrid assay

The BMC4 gene was PCR amplified (Mills-Lujan and Deom, 2010), using primers BMCTVNcoF and BMCTVC4BamR. The PCR product was digested with *NcoI* and *BamHI* and cloned into the multiple cloning site of the pGADT7 as described in Chapter 4. BMC4 interactions with AtSKs in yeast were determined using the Matchmaker Two-Hybrid System 3 (Clontech, Mountain View, CA) and the Galacto-Star System (Applied Biosystems Inc., Foster City, CA) as described in Chapter 4.

Bimolecular fluorescence complementation

The BMC4 gene was amplified as above, using primers BMCTVNcoF and BMC4-EYFP 3R, cloned into pBSKS+, subcloned into pSAT1-cEYFP-N1 vector (Citovsky et al., 2006) using

NcoI and *BamHI* restriction sites, and subsequently subcloned into the pPZP-RCS2-bar vector, as described for BCTV C4 in Chapter 4. The resulting pPZP-RCS2-BMC4-cEYFP clone was subsequently transformed into *Agrobacterium tumefaciens* strain LBA4404. Fusion proteins were transiently expressed in *N. benthamiana* CFP-H2B, and examined by confocal microscopy as described in Chapter 4.

Results

Beet mild curly top virus C4 protein interacts with multiple AtSK family members

The C4 protein of BCTV was shown to interact with sg2 AtSKs in a yeast two-hybrid assay (Piroux et al., 2007; Chapter 4). As previously mentioned, sg2 AtSKs are negative regulators of the BR signaling pathway (Ryu et al., 2007), and other AtSK family members have also been implicated in BR signaling (De Rybel et al., 2009; Yan et al., 2009). The interaction between BMC4 and four AtSK family members was tested using a yeast two-hybrid assay. BMC4 interacts with the three sg2 AtSKs as well as AtSK41 (Fig. 5.1A).

Since BMC4 lacks the putative phosphorylation site, Ser at residue 49, identified as important for interaction between C4 and sg2 AtSKs (Fig. 4.1 and Fig. 4.4), we decided to investigate whether the kinase domain on sg2 AtSKs was also important for the interaction between BMC4 and sg2 AtSKs. The AtSK21 kinase dead mutant, AtSK21-KD, cannot phosphorylate known substrates (Zhao et al., 2002; Ryu et al., 2007). The BMC4 interacted weakly with the sg2 AtSK kinase dead mutants in yeast two hybrid assays (Fig. 5.1B). This

suggests that both C4 and BMC4 interacted strongly with the same region of the sg2 AtSKs, which involves the kinase domain.

BMC4 interacts with subgroup 2 AtSKs, AtSK12 and AtSK41 in planta

Bimolecular fluorescence complementation (BiFC) was used to determine if the interactions observed in the yeast two-hybrid assay also occurred *in planta*. The BMC4 protein was fused to the C-terminal portion of EYFP (BMC4-cEYFP), as this fusion did not significantly alter the function of the C4 gene (Figure S 4.1). The BMC4-cEYFP fusion protein was co-agroinfiltrated with AtSK-nEYFP fusion proteins into *Nicotiana benthamiana* CFP-H2B. Transgenic *N. benthamiana* plant line CFP-H2B constitutively expresses cyan fluorescent protein (CFP) fused to histone 2B, which localizes to the nucleus (Martin et al., 2009) and served as a spatial reference. Sonchus yellow net virus protein fusions, N-nEYFP and P-cEYFP, which co-localize with the CFP-H2B reference signal in the nucleus (Fig. 4.4A and Fig. 5.2A), and GST-nEYFP co-agroinfiltrated with BMC4-cEYFP were used as positive and negative controls (Fig. 5.2A), respectively.

BMC4 interacted with all sg2 AtSKs, primarily at the plasma membrane, but also in the nucleus (Fig. 5.2A and B), similar to what was seen of C4/AtSK interactions (Fig. 4.4). The BMC4 interaction with sg2 AtSK-KD mutants was weak, but detectable, while the interaction between BMC4 and gain of function sg2 AtSK mutants (AtSK-GF) was similar to that of wild type sg2 AtSK/BMC4 interactions (Fig. 5.2B). This indicates that the kinase domain on sg2 AtSKs is important for the interaction with BMC4. As with C4, an interaction between BMC4 and AtSK13 or LRR-RLK was not detected (Fig. 5.2). However, unlike C4, BMC4 also

interacted with AtSK12, and more weakly with AtSK41 (compare Fig. 5.2 to Fig. 4.4), suggesting that BMC4 function could differ from that of C4.

Discussion and future perspectives

It cannot be assumed that the C4 genes of all curtoviruses have the same function. Limited evidence suggests that the C4 gene of BCTV and BSCTV have different functions, as the first has been implicated in disrupting BR signaling (Mills-Lujan and Deom, 2010), whereas the latter may regulate the function of RKP, a ubiquitin E3 ligase involved in cell cycle regulation (Lai et al., 2009). At the genomic level, BCTV shares 79.6% and 83.2% sequence similarity with BMCTV and BSCTV, respectively. However, the sequence similarity for the C4 protein is lower, with BMCTV C4 and BSCTV C4 sharing 63.3% and 72.5% sequence similarity to BCTV C4, respectively. Two major factors prompted us to investigate the role of BMC4 further. First, the low sequence similarity shared between C4 and BMC4, and second, the lack of the putative phosphorylation site, residue Ser49 (Fig. 4.1), found to be important for the function of BCTV C4 (Chapter 4). Our results show that, like C4, BMC4 interacts with sg2 AtSKs possibly through the sg2 AtSKs kinase domain, despite the lack of Ser49 on BMC4. One possible explanation is that both BMC4 and C4 interact with the sg2 AtSKs at the kinase domain, but are not phosphorylated by the kinases. BMC4 has an Asn at residue 49, and the structure of the non-polar side chain on Asn is very similar to that of a non-phosphorylated Ser. Thus, structurally Asn could substitute for Ser at the BMC4/C4 binding pocket of sg2 AtSKs. Alternatively, the BMC4/sg2 AtSKs interaction might not be biologically significant, and thus, not lead to downstream effects. This hypothesis can be tested by several methods. The first is to

determine if BMC4 induces a C4-like phenotype, either by inoculating BMC4 onto *N. benthamiana* using a PVX-vector, or using transgenic lines that express BMC4 from an inducible promoter. We are currently in the process of screening transgenic IPBMC4 lines (data not shown). However, the induction of a phenotype may not necessarily correlate with BMC4 altering the BR signaling pathway through its interaction with AtSKs. Establishing whether BMC4 induces changes in the phosphorylation status of BES1, in the transgenic plant lines, is one way in which this could be determined.

It is also plausible that the effects that C4, and possibly BMC4, have on BR signaling are not the real cause behind the gross developmental phenotype induced by C4. In previous work it was proposed (Chapter 4) that phosphorylated C4 could interact with targets downstream of the C4/sg2 AtSK interaction, which might not be the case for BMC4, as it lacks the Ser49 residue. If this were the case, identifying the host factors that interact with pC4 would open a whole new area in the study of C4/BMC4. Alternatively, the interaction between BMC4 and other AtSK family members may be countering the effects of the BMC4/sg2 AtSKs interaction. We showed here that BMC4 interacts with AtSK12, and to a lesser extent with AtSK41 *in planta*. This still leaves the possibility that BMC4 interacts with AtSK11, subgroup 3 AtSKs, and AtSK42. The function of AtSKs outside of sg2 remains largely unknown, and understanding the biological significance of the interaction between BMC4 and the other kinases could help shed some light into the function of these other AtSK family members.

We are only beginning to understand the function of the curtovirus C4 protein. Many of the arguments made in this Chapter have yet to be determined, but some of the groundwork for these experiments has already been done. It will be interesting to compare the mode of action of the C4 protein of BMCTV and BCTV, two closely related viruses, and how this relates to the

overall infection phenotype. In addition, the study of C4/BMC4 could prove to be useful for understanding endogenous plant processes.

Figures

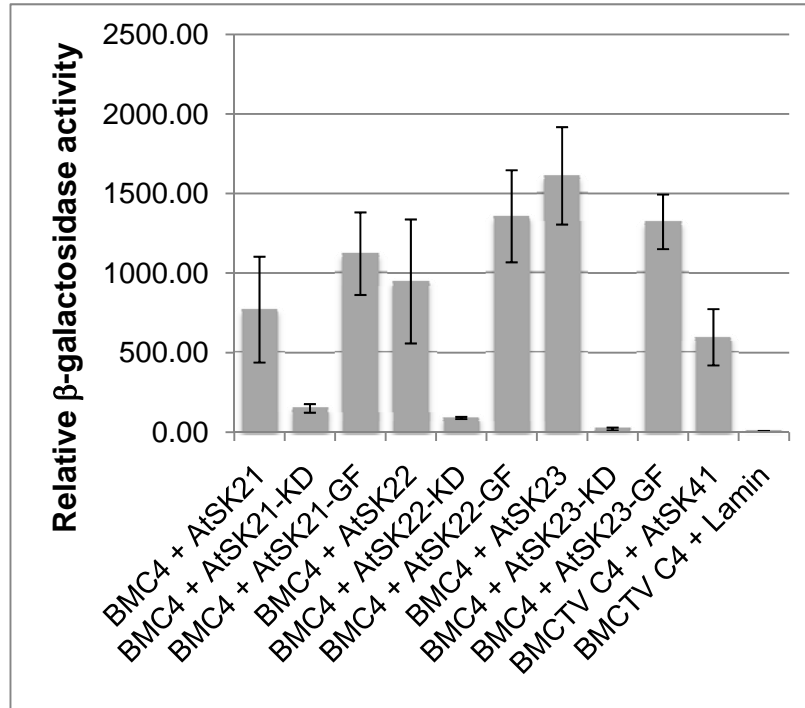


Fig. 5.1 BMC4 interacts with subgroup 2 AtSKs and AtSK41. Comparison of the interaction between BMC4 and wild type sg2 AtSKs (AtSK21, AtSK22, and AtSK23), kinase dead (KD) and gain-of-function (GF) mutants of sg2 AtSKs, and AtSK41. B-galactosidase activity was quantified using duplicate samples from three independent yeast transformants, and is expressed as relative to the T-antigen/p53 positive control. Interaction between BMC4 and Lamin used as a negative control.

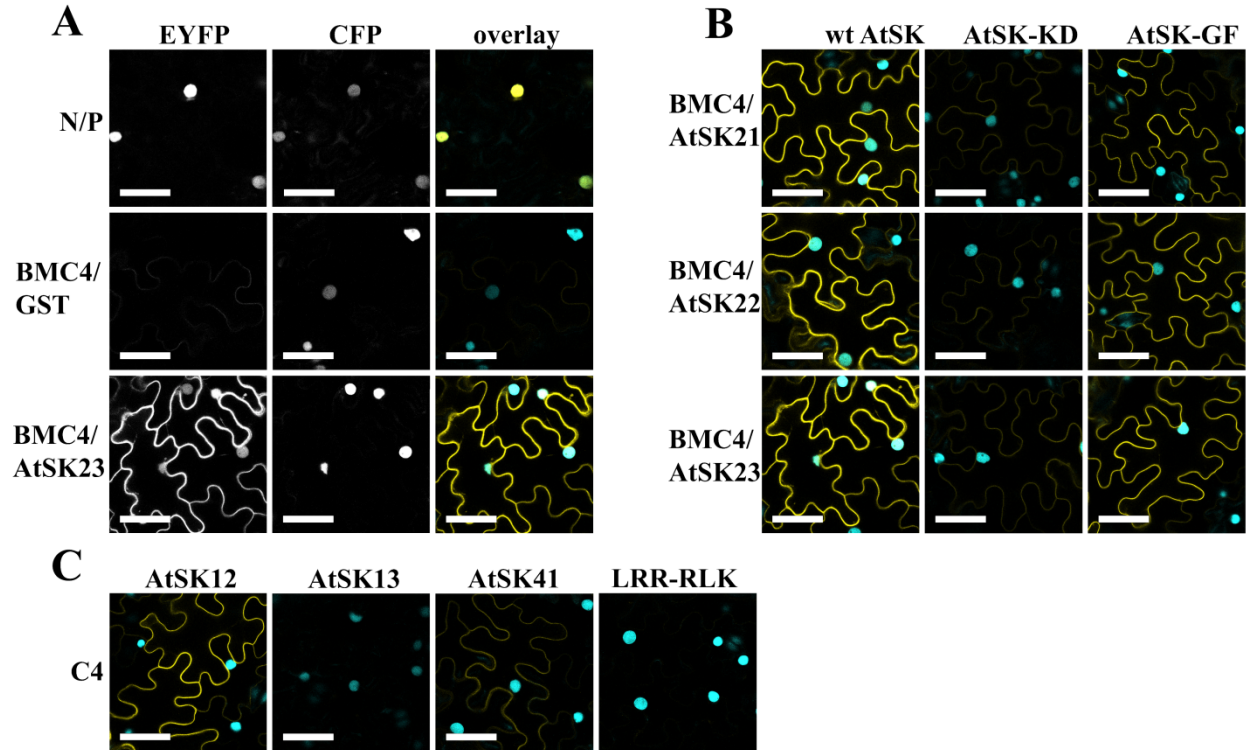


Fig. 5.2 BMC4 interacts with subgroup 2 AtSKs, AtSK12 and AtSK41 *in planta* using bimolecular fluorescence complementation (BiFC). Confocal micrographs showing differential interactions between BMC4 with AtSKs and sg2 AtSK mutants co-infiltrated into *N. benthamiana* CFP-H2B lines. Reconstituted EYFP, marker line CFP and overlay of both fluorescent signals are shown in (A). Only the overlay of EYFP and CFP signals are shown in (B-D). (A) EYFP signal (yellow) from SYN-V-N and SYN-V-P positive control interaction colocalizes with fluorescence from CFP-H2B (blue) in the nucleus, as seen in the overlay (upper row). No EYFP signal was detected from the GST/C4 negative control (middle row). AtSK23 and C4 interact at the plasma membrane and in the nucleus (yellow; bottom row). (B) BMC4 interacts with AtSK21, AtSK22 and AtSK23 and gain of function mutants (AtSK21-GF, AtSK22-GF and AtSK23-GF) at the plasma membrane and in the nucleus (yellow, first and third columns, respectively), and interacts very weakly with kinase dead (KD) mutants (second column). Only CFP-H2B fluorescence is visible due to the lack of interaction between BMC4 and AtSK21-KD, AtSK22-KD, and AtSK23-KD. (C) BMC4 interacts with AtSK12 and AtSK41, at the plasma membrane, but does not interact with AtSK13 or LRR-RLK. Scale bar = 50 μ M

Tables

Table 5-1 Primers used for cloning and mutagenesis of BMCTV C4

Primer name	Primer sequence	Mutant or cloning application
BMC4L48S FOR	CTG CAT CCG ATG TCA AAC AAT ACA TCG	BMCTV C4L48S
BMC4L48S REV	CGA TGT ATT GTT TGA CAT CGG ATG CAG	
BMC4N49S FOR2	CAT CCG ATG TTA AGC AAT ACA TCG AGA	BMCTV
BMC4N49S REV	TCT CGA TGT ATT GCT TAA CAT CGG ATG	C4N49S
BMC4N49T FOR	CAT CCG ATG TTA ACC AAT ACA TCG AGA	BMCTV C4N49T
BMC4N49T REV	TCT CGA TGT ATT GGT TAA CAT CGG ATG	
BMC4-5Cla	CCGATCGATAACAATGGGTCTCTGCATCTCCACG	Cloning BMC4 into PVX vector
BMC4-3EcoRV	CCGGATATCTTAATGCTTCTGCTGCTGCAT	
BMCTVNeoF	CCGCCATGGGTCTCTGCATCTCCACG	Cloning BMC4 into pSAT vector
BMC4-EYFP 3R	CCGGGATCCcATGCTTCTGCTGCTGCATCAT	
BMCTVNcoF	CCGCCATGGGTCTCTGCATCTCCACG	Cloning BMC4 into pGADT7
BMCTVC4BamR	CCGGGATCCTTAATGCTTCTGCTGCTGCAT	
BMC45	CCGCTCGAGAACAATGGGTCTCTGCATCTCCACGCC	Cloning BMC4 into pER10
BMC43	GGACTAGTTTAATGCTTCTGCTGCTGCATCATTAGC	

CHAPTER 6

CONCLUSIONS

Viruses are being used more frequently to study many different endogenous plant processes. The C4 protein of BCTV offers an excellent model for studying cell cycle regulation, cell differentiation, and development in plants. The overall goal of the project, of which this dissertation is part, is to determine the role of the C4 oncogene. Of course the aim of this goal is beyond the scope of this dissertation. Herein, we set the foundation for possible pathways and processes altered by C4 and future directions and avenues of research.

The first objective of this dissertation was to carry out a detailed phenotypic characterization of the effects of C4 expression on plant development. For this we generated transgenic *Arabidopsis* plants expressing the C4 gene under regulatory control of an inducible promoter (line IPC4-28) and used several microscopy approaches to examine the gross and cellular effects of C4 expression. We showed that the increase in cell division induced by C4 was not localized, but rather occurred in all tissue types examined. We also determined that, in addition to increasing cell division, expression of C4 led to loss of meristematic function, cell differentiation, and cell identity. Induced seedlings failed to develop shoot and root apical meristems, vascular tissue, specialized organs, and had disorganized tissue layers.

The second objective was to determine the mechanism of C4 function. Our approach was two-pronged. The first was to pinpoint key amino acid residues for the C4 protein's function using site-directed mutagenesis of C4. One putative phosphorylation site, Ser49, was identified

as important, since mutations to both Ala (C4S49A) and Glu (C4S49E) abolished the function of C4, whereas substitution to Thr (C4S49T) restored the protein's function. The importance of this mutation was further confirmed using transgenic Arabidopsis plants. The phenotype of seedlings expressing C4S49T was reminiscent of the C4-induced phenotype, whereas the expression of C4S49A had no obvious effect on seedling development.

In a second approach, we investigated whether the recently discovered interaction between C4 and AtSK21 and AtSK23 *in vitro* (Piroux et al., 2007), was biologically significant *in planta*. We hypothesized that the interaction between C4 and AtSK21, a known negative regulator of BR signaling, would lead to misregulation of the BR signaling pathway. To test this hypothesis we compared the expression levels of BR signaling pathway target genes, *CDP*, *DWF4* and *SAUR-15*, in the presence and absence of C4. In the absence of BR, AtSK21 hyperphosphorylates and inactivates BES1 and BZR1 (Kim et al., 2009). When BR is present, it binds to BRI1 receptor kinase, initiating a signaling cascade that results in the activation of BSU1 phosphatase, and subsequent inactivation of AtSK21 (Kim et al., 2009). The inhibition of AtSK21 allows the BZR1 and BES1 transcription factors to be dephosphorylated, accumulate in the nucleus, and bind to BR-target gene promoters, resulting in the down-regulation of *CPD* and *DWF4* and the up-regulation of *SAUR-15* (Li and Deng, 2005; Belkhadir and Chory, 2006; Li and Jin, 2007). Our results indicated that the expression of C4 had a negative effect on the function of AtSK21, and presumably other sg2 AtSKs. In the presence of C4, the expression of *SAUR-15* was up-regulated and *CPD* and *DWF4* were down-regulated, suggesting that AtSK21 was inactive. This was in accordance with our hypothesis. We then determined if the C4/AtSK interaction occurred *in planta*. Using BiFC, we found that C4 interacts specifically with all sg2 AtSKs, and that the C4S49A mutant does not. This led us to speculate that C4 could be a

substrate for sg2 AtSK kinase activity. To test this we generated kinase dead mutants, used BiFC to determine if they interacted with C4, and found that C4 does not interact with the sg2 AtSK-KD mutants. From these results we concluded that C4 inhibits the kinase activity of sg2 AtSKs, and is likely a substrate of sg2 AtSKs. Finally, because the effects of C4 expression are greater than the triple sg2 AtSK knockout mutant (Vert and Chory, 2006), it is possible that other host factors interact with phosphorylated C4 (pC4) downstream of the C4/sg2 AtSK interaction.

Based on the data presented herein, we propose a model (Fig. 6.1) by which C4 inhibits the function of sg2 AtSKs. The C4 protein interacts with sg2 AtSKs at the plasma membrane and in the nucleus, resulting in the activation of C4 protein through phosphorylation of amino acid residue Ser49 on C4 by the sg2 AtSKs. This allows active non-phosphorylated BES1 and BZR1 to accumulate, leading to BR response-like changes in gene expression. In addition to competing with BZR1/BES1 for phosphorylation by sg2 AtSKs, C4 sequesters the normally nuclear/cytoplasmic localized sg2 AtSKs at the plasma membrane, away from the sg2 AtSK substrates BES1 and BZR1, further inhibiting the sg2 AtSKs' activity on the transcription factors. Activated pC4 protein presumably interacts with unidentified downstream targets, leading to additional cellular and developmental responses.

However, further evidence is needed to definitively conclude that C4 is a substrate of sg2 AtSKs, and that pC4 is a substrate for downstream targets. To this effect, we have generated transgenic Arabidopsis plants of a C-terminally StrepII-tagged C4 gene under the regulatory control of an inducible promoter (line IPC4-SII; data not shown). The functionality of C4-StrepII was initially tested in *N. benthamiana* using the PVX-vector and no adverse effects were seen on C4 function (data not shown). Preliminary tests show that induced IPC4-SII lines show a C4-expression phenotype (data not shown). Initial applications for line IPC4-SII are two-fold. First,

C4-StrepII can be purified and analyzed by mass spectrometry to determine if Ser49 is actually phosphorylated *in planta*. Second, C4-StrepII can be used to confirm the presence of C4/sg2 AtSK complexes in *Arabidopsis*, using co-immunoprecipitation. Furthermore, C4-StrepII could also be used to identify additional host proteins that interact with C4, using a combination of pull-down assays with mass spectrometry. This could be done by either inoculating PVX-C4-SII onto *N. benthamiana*, or inducing *Arabidopsis* line IPC4-SII. This approach could potentially identify downstream targets of the C4/sg2 AtSK interaction, or other host factors that interact with C4 independent of the sg2 AtSK pathway.

Determining the effects of BMC4 on plant development could also shed light on the importance of the Ser49 residue of C4, which corresponds to Asn49 on BMC4. It remains to be determined if BMC4 is capable of inducing the same phenotype as C4 on either *N. benthamiana* or *Arabidopsis*. To this effect, the BMC4 gene, under the control of an inducible promoter, has been transformed into *Arabidopsis*, and initial screening is currently under way for transgenic plants (data not shown). A BMC4 mutant, which has Ser at position 49 (BMC4N49S), has been generated and placed in the PVX-vector for future comparison with PVX-BMC4 in *N. benthamiana*. I hypothesize that BMC4 does not induce a severe C4-like phenotype. The rationale behind this hypothesis is that even though BMC4 is capable of interacting with sg2 AtSKs, this interaction does not lead to phosphorylation of BMC4 nor an interaction with downstream targets. This hypothesis is one that could be tested in the future.

Finally, we also showed that the endogenous application of specific plant hormones alters the effects of C4 expression on transgenic IPC4-28 lines. The addition of BR and abscisic acid weakly rescued the C4-induced phenotype, whereas induced seedlings were hypersensitive to gibberellic acid (GA) and kinetin. The weak recovery of the C4-induced phenotype by BR is

possibly due to the fact that *sg1* AtSKs and AtSK31, which do not interact with C4, are responsible for the BR-response. This is an additional hypothesis that can be tested in the future. More interesting would be to delve further into the hypersensitivity of C4-expressing seedlings to GA and kinetin, since this could be an alternative pathway that C4 is misregulating. Alternatively, the hypersensitivity of C4-expressing seedlings to GA and kinetin could be due to the great amount of cross-talk between hormone pathways.

Even though the work presented herein moves us closer to a better understanding of C4 function, it is evident that much still remains unknown. C4 has proven to be an important tool for understanding plant development. Its potential application in understanding further aspects of BR signaling is clear. C4 could additionally be central in finding a link between BR signaling and cell cycle regulation. Comprehending how C4 expression leads to increased cell division could translate into further knowledge about cell cycle regulation in plants. The practical applications behind understanding the mechanism by which C4 induces cell division could be used to generate plants with increased biomass. And of course, understanding the role of viral genes during infection is needed to design better control strategies to manage geminivirus-induced disease.

Figures

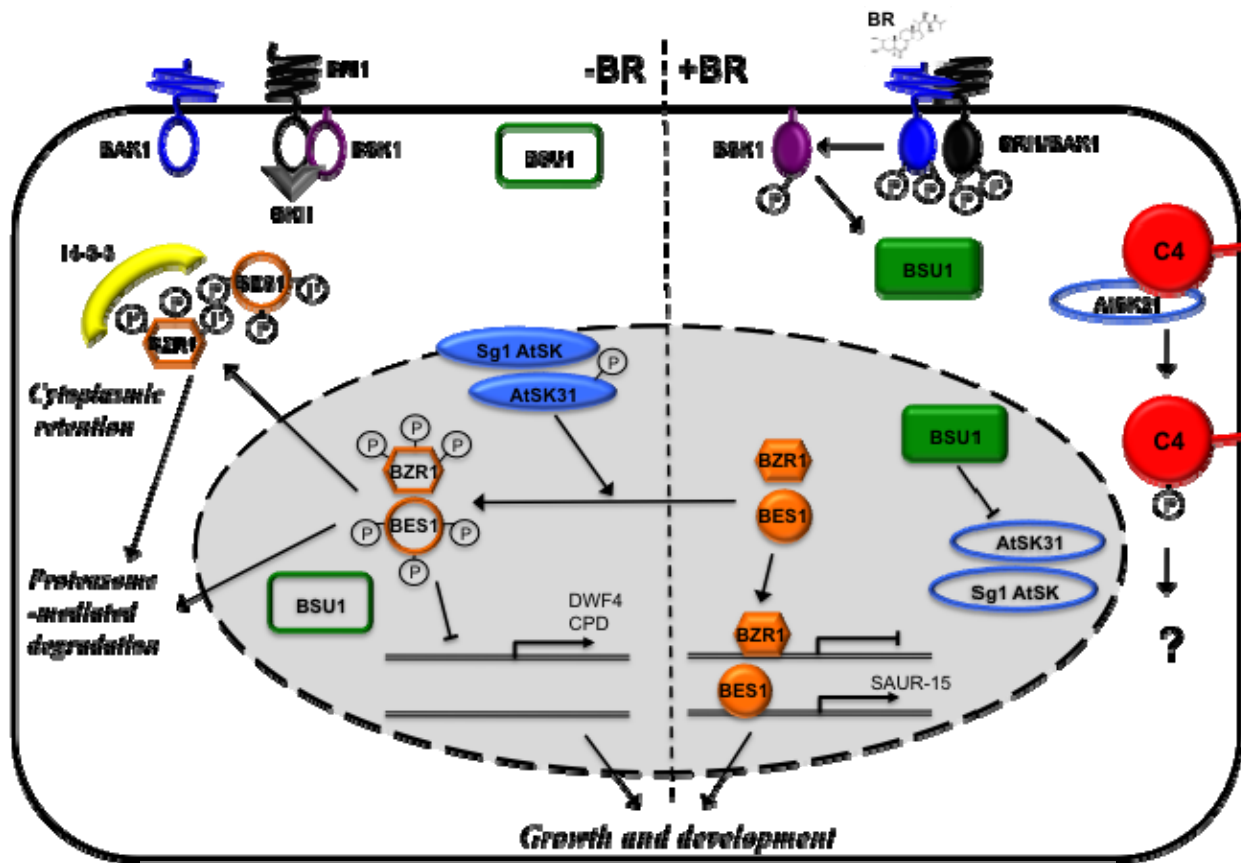


Fig. 6.1 Proposed model of C4 protein function. C4 protein interacts with sg2 AtSKs at the plasma membrane, and sequesters sg2 AtSKs from interacting with BZR1/BES1 transcription factors. In addition, C4 protein is a substrate for sg2 AtSKs, and competes with BZR1/BES1 transcription factors for phosphorylation by the sg2 AtSKs. This allows non-phosphorylated BZR1 and BES1 to accumulate and bind to their target promoters, resulting in a response reminiscent of BR-response signaling cascade (see **Fig. 2.2**). C4 is phosphorylated on amino acid residue Ser49 by sg2 AtSKs. The phosphorylation of C4 results in the protein's activation and possible interaction with downstream host targets. In the presence of C4 protein and absence of BR, sg1 AtSKs and AtSK31 remain active, but become inactivated in the presence of BR (see **Fig. 2.2** for BR response). Filled objects indicate an active state, open objects an inactive state. Modified from (Kim et al., 2009).

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