Light Induction of Somatic Embryogenesis and Dark Germination of *Arabidopsis thaliana* (L.) Heynh.

BY

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ABSTRACT

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The ability to form embryos from somatic cells or somatic embryogenesis (SE) is a form of totipotency present in plants. Somatic embryogenesis has been shown in past literature to have varied response to light, but the mechanism of light effect is poorly understood. This work attempts to delve deeper into the understanding of the effects of light stimulation of SE. Somatic embryogenesis of *Arabidopsis thaliana* in the visible region was mainly induced by red light (660 nm). The red light receptor *PHYTOCHROME E* (*PHYE*) was the only *PHY* mutant that reduced SE. Transcriptional analysis of *phyE* and darkness showed several hundred fold reduction in *CONSTITUTIVE PHOTOMORPHIC DWARF* (*CPD*) expression, a rate limiting enzyme in brassinosteroid (BR) biosynthesis. Application of BR partially rescued *AGAMOUS LIKE 15* (*AGL15*) expression, a key SE gene, under darkness and in the *phyE* mutant. The results support a model where red light acts through PHYE and subsequently brassinosteroid signalling to induce SE through AGL15.

Agriculturally important plants usually germinate in soil under darkness. Dark germination is seldom researched from a developmental angle. One reason is the lack of dark specific germination mutants. The *WUSCHEL RELATED HOMEOBOX 7* (*WOX7*) mutant of Arabidopsis Columbia (Col-0) germinates 100% under light, but nearly 0% under darkness. During dark germination *WOX7* expression was not detected suggesting that the *wox7* phenotype likely occurs from seed setting effects. Germination tests revealed that Col-0 requires nitrate to germinate in the dark. Nitrate induced dark germination was enhanced by cytokinin (CK) and co-application rescued *wox7*. Nitrate and CK induced dark germination was found to be upstream of *PHYTOCHROME B* (*PHYB*). Dark germination seems to be a phenomenon that arises from nitrate activation of PHYB in place of light and this effect in part acts through nitrate modulation of cytokinin, which indirectly requires WOX7.

INTRODUCTION

Somatic embryogenesis is the formation of embryos from somatic cells is a possibility that arises from plant totipotency. Totipotency or the ability of a cell to differentiate into any cell type of an organism is typically restricted to zygotes in complex organisms. Understanding how organisms develop is the goal of developmental physiology. Thus this study aims at elucidating how somatic embryos develop from somatic tissue. In particular this study examines the regulatory effect of light on SE induction; the series of physiological events that eventually lead to SE.

Visible light is one induction factor that past studies have failed to reach consensus on (reviewed by Gaj 2004). Even in the same plant, *Arabidopsis thaliana*, there exist contradictory results (most are not recent, over 15 years old). Unlike somatic embryogenesis, plant and light interaction is rich with literature. Light reception mechanics have solid models and well characterized, available mutants. Furthermore, numerous transcription studies have identified candidate genes that respond downstream to light signals. Light sources of higher quality have also become more affordable. Thus an opportunity for more in depth research (than 15 years ago) is possible.

Scientific progress often arises from putting effort in pursuing chance discoveries that become opportunities. The discovery of the dark germination phenotype of *wox7* is one such chance. Dark germination is more applicable to agronomically important field conditions, but rarely pursued academically as popular interest lies with light germination. The lack of even basic literature in the model dicot, Arabidopsis, specifically the genome sequenced Col-0 presents a need to establish a starting point of study. Discovering the difference in entry points under darkness compared to light will also further elucidate where signalling stops and physiological changes causing germination occurs. Finding the factors that signal germination under darkness is the aim of this side project.

LITERATURE REVIEW

Plant Embryogenesis

Embryogenesis is the process where a single cell differentiates into a new generation of the parent organism. In flowering plants like Arabidopsis, a single zygote cell arises from sexual reproduction by the gametes; pollen (microspores) formed in the anthers and the megaspores formed in the ovules (reviewed by Berger *et al.*, 2008). The megaspore undergoes double fertilization by the microspore; forming the endosperm with the central cell and the zygote with the egg cell. Embryogenesis begins when the zygote undergoes asymmetric division to form the apical and basal regions (Fig L.1).

Basal identity genes *WUSCHEL RELATED HOMEBOX 8 and 9* (*WOX8/9*) (Fig L.1) are required for polar division of the zygote (Wu *et al.*, 2007). *WOX8/9* specifies the expression of *WOX2* in the upper apical domain, which affects proper planar cell division until the 16 cell stage (reviewed by Lau *et al.*, 2012). Periclinal division in the apical region forms the 8 cell stage, where the apical domain is subdivided into upper and lower regions. The upper region expresses the shoot apical meristem (SAM) organization center (OC) "stem cell" maintenance gene *WUSCHEL* (*WUS*) at the 16 cell stage, while the lower hypophysis region expresses root apical meristerm (RAM) defining *PLETHORA 1* (*PLT1*). During this stage, the phytohormone auxin is transported to the apical region from the basal suspensor directed by PINFORMED (PIN) proteins (reviewed by Jenik *et al.*, 2007).

PIN proteins work in conjunction with *MONOPTEROS* (*MP*), an auxin response factor (*ARF5*), and *BODENLOS* (*BDL*), an auxin degraded *AUX* (*IAA12*) transcription inhibitor modulates auxin levels throughout the developing embryo (reviewed by Lau *et al.*, 2012). After apical directed auxin flow specifies the proto SAM, the auxin flow reverses to head to the basal end to specify the proto RAM. The RAM forms from the hypophysis as PLT activity localizes auxin maxima to specify the quiescent center (QC), the "stem cell" storage in the root, which is maintained by *WOX5* similar to how *WUS*

maintains the SAM stem cells (reviewed by Jenik *et al.*, 2007). After developing a SAM and RAM the embryo, which at the time is visibly amorphous (globular), begins to develop distinct shoot organs (Fig L.2).

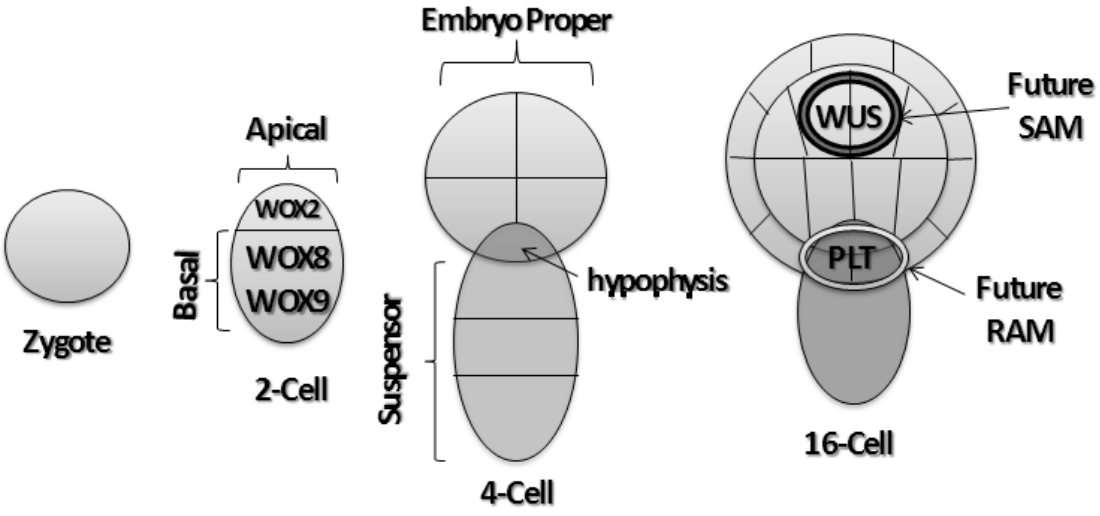

Fig L.1 Early Embryogenesis in Arabidopsis thaliana

The zygote divides asymmetrically into an apical cell expressing *WOX2* and a basal cell expressing *WOX8/9*. The apical cell forms the embryo proper, while the basal cell forms the hypophysis and suspensor. After the apical domain divides into 16 cells, *WUS* is expressed in the future SAM region, while *PLT* is expressed in the hypophysis that forms the RAM.

The shoot begins to develop cotyledons (embryonic leaves) from cells differentiating from the SAM. SAM cell differentiation requires *SHOOT MERISTEMLESS* (*STM*), a class I *KNOTTED-like HOMEOBOX* (*KNOX*) transcription factor (reviewed by Lau *et al.*, 2012). *STM* cross-talks with *CUPSHAPED COTYLEDON* (*CUC*) genes coordinating temporal-spatial expression allowing separation of the cotyledons making the embryo visibly heart shaped (Fig L.2).

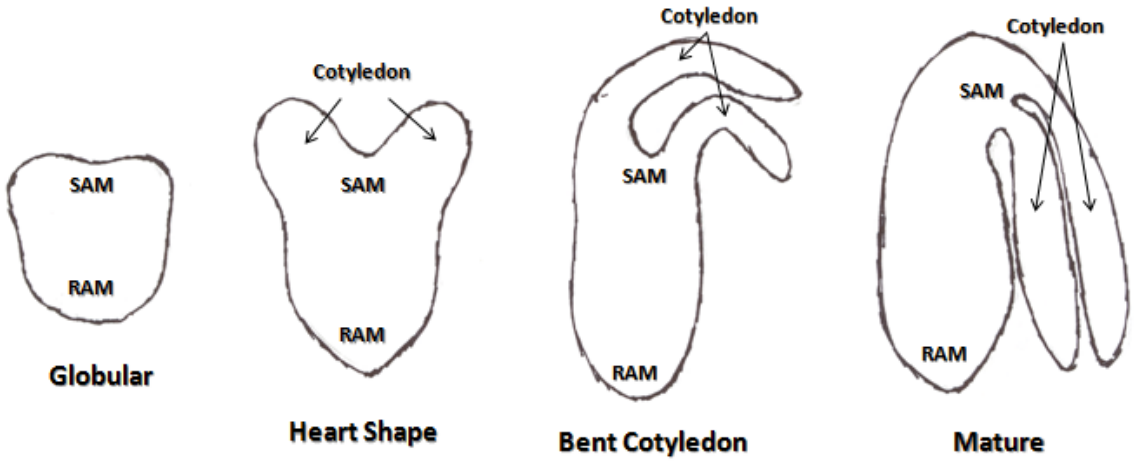

Fig L.2 Late Embryogenesis in Arabidopsis thaliana
After the SAM forms the embryo begins to gain distinctive morphology from the globular shape it began with. The embryo becomes visibly heart shaped as the SAM produced cotyledons extend and gradually bend due to restraints of the embryo sac. After growing to full size the mature embryo desiccates (reviewed by Jenik *et al.*, 2007).

Outgrowth of the cotyledons eventually is restricted by the surrounding embryo sac and the cotyledons become visibly bent (Fig L.2). Completion of cotyledon development can be considered the end point of embryogenesis after which *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) proteins prepare the embryo for survival by storing germination factors and establishing desiccation tolerance (reviewed by Jenik *et al.*, 2007).

Shoot development is not a fixed fate of embryo SAM derived cells. Loss of class III *HOMEODMAIN-LEUCINE ZIPPER* (*HD-ZIP III*) gene function, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), result in loss of development of shoot structures (reviewed by Lau *et al.*, 2012). Loss of function of the *WUS* related *TOPLESS* (*TPL*) gene can at higher temperatures cause root development from the apical pole of the embryo. Apical fate differentiated cells are capable of differentiating into root structures because plant cells possess totipotency.

Plant Cell Totipotency

Totipotency is the ability of a cell to produce all differentiated cell types available to an organism. Differentiated non-germ (somatic) cells typically lose totipotency, although they may be pluripotent being able to produce a subgroup of cell types i.e. stem cell. Certain somatic plant cells are capable of regaining totipotency (reviewed by Verdeil *et al*., 2007).

Discovery of plant totipotency predates modern science when plant growers found that whole plants could be regenerated from cuttings; now known as vegetative propagation (reviewed by Geneve 2001). Vegetative propagation prior to the discovery of phytohormones (1930s) relied on the natural wound regeneration ability of select cultivars, as well as co-culture with microbes that enhanced response (reviewed by Preece 2003). Microbial effects were later found to be due to release of phytohormone analogs. The phytohormone auxin was identified as the major endogenous inducer of vegetative propagation and wound regeneration (reviewed by Swingle 1940).

Plant regeneration is preceded by the formation of callus tissue at the site(s) of wounding. Callus formation is also induced by species dependent combinations of the phytohormones auxin and cytokinin (reviewed by Preece 2003). By adjusting the ratio of auxin to cytokinin callus tissue can be induced to form shoots or roots, thus callus cells can be considered totipotent. Callus can arise from almost all living plant cell types, but only some can be induced to become embryos, such cells are competent for SE. Competent callus cells can respond to environmental cues and become determined (cell fate switch) to form embryos (reviewed by Verdeil *et al.*, 2007). This type of plant regeneration is referred to as somatic embryogenesis.

Somatic Embryogenesis in *Arabidopsis thaliana*

The current model dicot is *Arabidopsis thaliana*. Using Arabidopsis as a model has been well established in many fields of plant study because Arabidopsis has a short life cycle, a small genome and is fully sequenced.

Although Arabidopsis is a model species it is a relatively late comer to the study of embryo formation from somatic cells or SE due to recalcitrance. Recalcitrance is the resistance to exhibiting behaviour; in this case, forming somatic embryos. Initial attempts of SE in Arabidopsis followed the established carrot cell suspension system (O'Neill and Mathias, 1993; Kaldenhoff *et al.*, 1994). Suspension cultures were taken from leaf protoplasts or shoot callus, induced on 2, 4-D media and matured in hormone free media. SE induction is the process where embryogenic competent cells become determined to form somatic embryos (reviewed by Namasivayam 2007). Determination is the commitment to a developmental process resulting in a phenotype; in this case somatic cells becoming somatic embryos. Visible embryo formation, the amount of which determines SE response, from determined cells occurs on maturation medium, which is typically hormone free as per the cell suspension system (Kaldenhoff *et al.*, 1994). Cell suspension was difficult to work and yielded low SE and was displaced by the immature zygotic embryo systems.

Wu *et al* (1992) demonstrated the ability of immature zygotic Arabidopsis embryos to undergo SE when induced on high auxin and low cytokinin hormone media followed by maturation on hormone free media. The procedure (Fig L.3) used in this thesis follows the above idea, but uses a custom 2, 4-D (auxin) containing, cytokinin-free media (Supplemental 4.B). In this system a somatic embryo is defined as an independent structure possessing independent vasculature and having an identifiable apical (SAM, cotyledons) and basal (RAM, hypocotyl) domain. The auxin in the media often causes aberration such as poly-embryos or fused embryos (reviewed by Namasivayam 2007). Both types are considered a single embryo. Only explants that form novel embryos are scored as embryogenic as all surviving explants form callus.

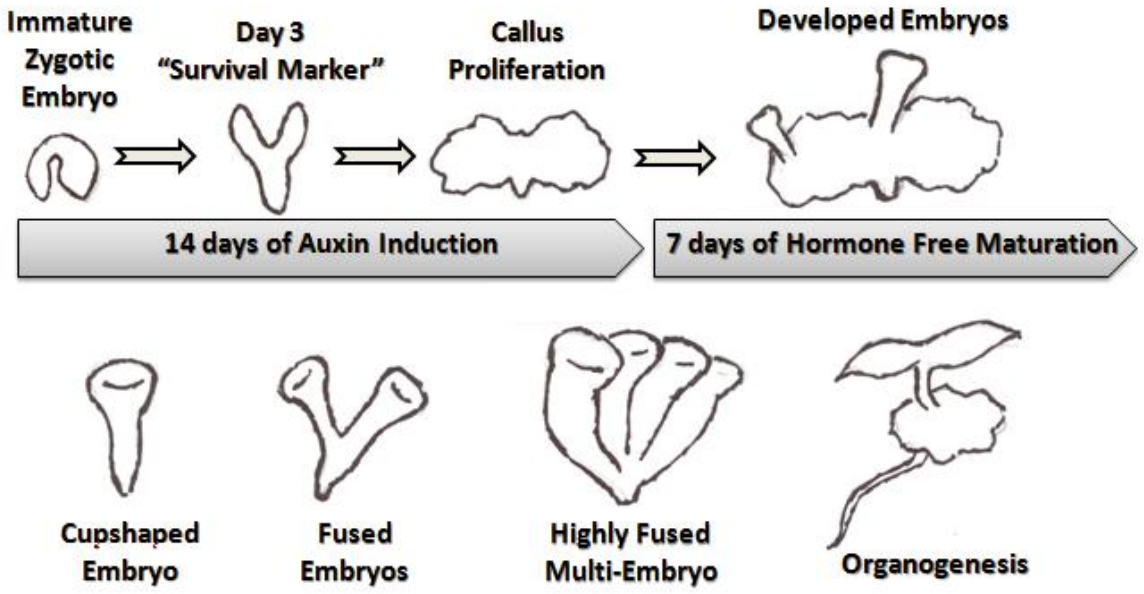

Fig L.3 Somatic Embryogenesis in Arabidopsis from Immature Zygotic Embryos Induced by Auxin

(Top) Almost mature, pre-desiccation embryo explants are placed on 2, 4-D medium for 14 days under continuous light. Cotyledons straighten by the 3rd day if explants survive. Callus is first visible by day 4 or 5, mainly on the apical tissues and proliferates until transfer to hormone free media. Hormone free conditions allows for embryo growth resulting in visible embryos. (Bottom) Some embryos have cup shaped cotyledons. Fused embryos are scored as one embryo. Organogenesis is when only either a shoot or root pole is initiated and is not considered SE.

Further work by Luo and Hoop (1997) found that SE response was highly dependent on explant stage and auxin concentration. In addition they also observed that somatic embryo development was similar to zygotic embryo development. Genes expressed in the developing zygotic embryo; *LEAFY COTYLEDON 1 (LEC1), ABSCISIC ACID INSENTITIVE 3 (ABI3)* and *FUSCA 3 (FUS3)* were found to be expressed during SE (Ikeda-Iwai M *et al.*, 2002). All three genes belong to a subgroup of B3 domain transcription factors that include *LEAFY COTYLEDON 2 (LEC2).*

Known SE Mutants in *Arabidopsis thaliana*

LEC1 and *LEC2* overexpression was found to cause SE formation on the apical portion of germinated seedlings (Lotan *et al.*, 1998; Stone *et al.*, 2001). Loss of function of a repressor of *LEC1* expression, the CHD3 chromatin remodelling factor *PICKLE* (*PKL*) results in spontaneous SE in roots (Ogas *et al.*, 1999). SE response was highly impaired in *lec1*, *lec2* and *fus3* mutants (Gaj *et al.*, 2005). *LEC1, LEC2, ABI3* and *FUS3* are all direct targets of the MADS box domain transcription factor *AGAMOUS-LIKE 15* (*AGL15*) which upregulates their transcription during SE (Zheng *et al.*, 2009). *AGL15* reduces SE response when knocked down, while ectopic expression caused SE formation in the shoots of seedlings similar to *LEC2* over expression (Harding et al., 2003).

AGL15 was observed by Karlova *et al.* (2006) to form a protein complex with SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1). SERK1 is a plasma membrane bound leucine rich repeat, receptor-like kinase (LRR-RLK), which has no ligand binding domain and dimerizes with other RLKs. *SERK1* expression is found in SE competent cells and overexpression increases SE response, while knock down does the opposite (Hecht *et al.*, 2001).

Probing AGL15 targets identified more SE related genes. Wang *et al.* (2004) found that *GIBERELLIN 2-OXIDASE 6* (*GA2ox6*), which decreases active gibberellic acid (GA), was up-regulated by AGL15. Constitutive expression of *GA2ox6* enhanced SE response, while GA application was suppressive. Zheng *et al.* (2009) further identified the AGL15 target *INDOLEACETIC ACID-INDUCED PROTEIN 30 (IAA30)*, an auxin response target, which lacks an auxin receptor binding site unlike its family members. *IAA30* overexpression again enhances SE competence (Zheng *et al.*, 2009). Expression of another auxin related gene, *TRANSPORT INHIBITOR RESPONSOR 1* (*TIR1*), an F-box protein that positively regulates auxin mediated proteasomal targeting, was down-regulated directly by AGL15 and *tir1* mutants had increased SE (Zheng *et al.*, 2011); however *tir1* somatic embryos were also found to have aberrant morphology (Su et al., 2009). Few auxin related factors have been isolated that strictly affect SE.

Ectopic over expression of the AP2/ERF transcription factor *PLT* family members, *BABY BOOM (BBM)* and *AINTEGUMENTA-LIKE 5 (AIL5)* resulted in enhanced SE response (Boutilier *et al.*, 2002; Tsuwamoto *et al.*, 2010). *PLT* members 1-4 are expressed in the root meristem region in response to auxin accumulation and maintain the "stem cell" identity as well as the root apical meristem (Aida *et al.*, 2004). Galinha *et al.* (2007) further demonstrated that high PLT levels prevent stem cell division while decreasing PLT levels first allow cell division and finally differentiation. While the activity of related members does not explain how *BBM* and *AIL5* specifically affect SE, it does suggest that "stem cell" identity factors are required for cell fate switch.

The shoot apical meristem "stem cell" maintaining homeodomain transcription factor *WUS* also improves SE response when expressed transiently using an inducible system (Zuo *et al.*, 2002). Gallios *et al.* (2004) replicated the constitutive 35S::*WUS* results where root derived somatic embryos formed but were developmentally impaired, although ectopic shoot formation developed without problems. Work by Su *et al.* (2009) further shows that auxin induction of *WUS* expression occurs during SE formation prior to morphogenesis. Auxin gradients formed in regions where somatic embryos would later arise, reminiscent of auxin maxima found in organ meristem regions.

Apart from *pkl*, known SE enhancing mutants were overexpression mutations. This suggests that embryogenesis is not a default developmental process that is being repressed until release by SE induction, but a change in the *de facto* genetic background through the transcriptome to confer a new cell fate. Transcription factors were the most common SE enhancing genes; embryo development regulators like the B3 family members or factors related to meristematic identity. Perhaps transcription factors that act as master regulators of transition between embryonic cell fates can, when improperly expressed at the right moment, trigger somatic cells to undergo SE. SE development, however, was reported to be disrupted by constitutive gene expression, suggesting that transient activity is required for genes involved in SE formation.

The mutants reviewed in this section were almost all of the gain of function (increased SE) variety (Fig L.4). Gain of function is easier to investigate because loss of function (reduced SE) because loss of function may be due to a secondary cause such as lowered stress tolerance or embryo viability, whereas gain of function suggests that the gene is directly involved in SE.

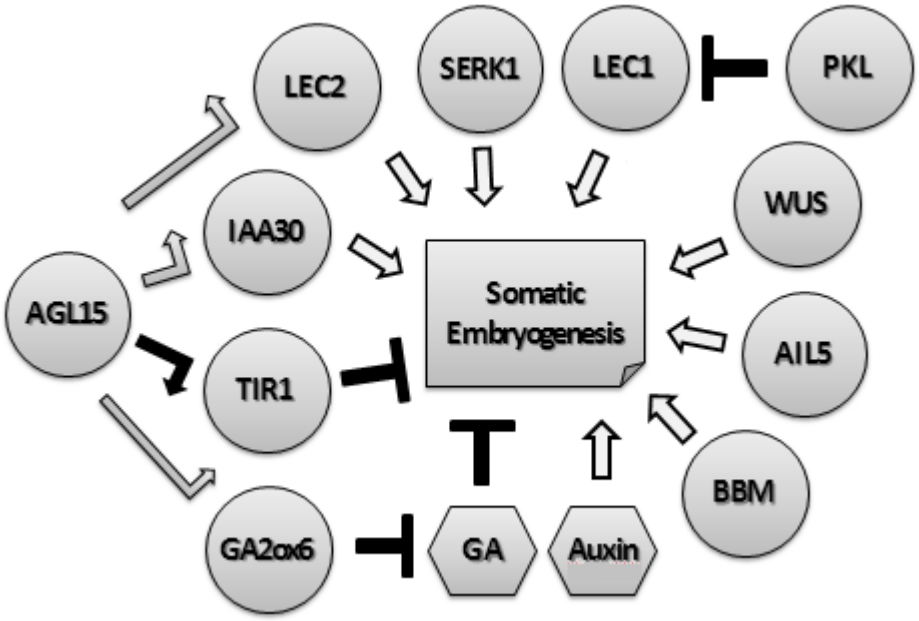

Fig L.4 Mutants Known to Affect Somatic Embryogenesis
Genes known to affect SE based on mutant phenotypes. Straight arrows represent up-regulation, block arrows represent inhibition, up-bent arrows represent transcriptional up-regulation and down-bent arrows represent transcription repression. Circles represent genes and their function, while hexagons are hormones. Gene names: *AGAMOUS LIKE 15 (AGL15), AINTEGUMENTA-LIKE 5 (AIL5), BABY BOOM (BBM), GIBERELLIN 2-OXIDASE 6* (*GA2ox6*), *INDOLEACETIC ACID-INDUCED PROTEIN 30 (IAA30), LEAFY COTYLEDON 1(LEC1), LEAFY COTYLEDON 1 (LEC2), PICKLE* (*PKL*), *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1), TRANSPORT INHIBITOR RESPONSOR 1* (*TIR1*), *WUSCHEL (WUS)*

The Role of Light in the Induction of Somatic Embryogenesis

The relation of light to the induction of SE is at the moment poorly characterized. In the model dicot Arabidopsis only two studies have tested the effect of light.

Gaj (2002) found that light had no effect on SE frequency (percentage of explants forming somatic embryos) in late stage immature zygotic explants, while light was repressive to early stage explants before the bent cotyledon stage. The second study by Kaldenhoff *et al.* (1994) using a cell suspension system found blue light (400-400 nm) promoted plantlet regeneration, while red light (600-700 nm) did not. However, the presence of light has been implicated to improve SE induction in other species.

Induction of SE in leaves of *Coffea canephora*, a light grown coffee species, required light and was almost completely repressed under darkness (De-la-Peña *et al.*, 2008). Darkness reduced SE response in leaf SE of *Doriataenopsis*, an orchid (Park *et al.*, 2010) and quince (D'Onorio *et al*., 1998). SE formation in spinach leaves showed that darkness (0 μmol $m^{-2}s^{-1}$) was inhibitory, while increasing light intensity up to 100 μmol $m^{-2}s^{-1}$ had increasing SE response (Milojević *et al.*, 2012). Darkness was found to have higher SE response than white light in carrot cell suspension culture (Michler and Lineberger, 1987)

Michler and Lineberger (1987) observed that red light, green light and darkness produced the highest SE response without significant difference between treatments. Takanori and Cuello (2005) in a retrial with light emitting diodes (LED) found that red light at 10 μmol $m^{-2}s^{-1}$ enhanced SE over darkness, but was not significantly different at lower or higher intensities. Park *et al* (2010) found that red and far red light were not significantly different and the SE response was enhanced by mixing red and far red light. D'Onorio *et al.* (1998) found that red light provided the highest SE response in quince leaves. Their mixed light experiments showed a decrease in red light induction by blue or far red light addition.

Blue light was found to inhibitory to SE in carrot (Michler and Lineberger, 1987), quince (D'Onorio *et al*., 1998) and agave (Rodríguez-Sahagún *et al.*, 2011). SE inhibition by blue light in carrot was found to increase with intensity and was specific to SE induction as blue light improved SE maturation (Takanori and Cuello, 2005).

In *Araujia sericifera*, a perennial creeping vine, the SE response of immature flower petals did not differ between long (16 h) or short (8 h) photoperiods (Torné *et al.*, 2001). Short days had reduced SE response when treated with far red end of day light, but long days were unaffected.

The general consensus from cross species studies is that light signalling affects SE. SE in most species was enhanced by red light reception, while blue was sometimes inhibitory. SE response similarities between distant species suggest that there is a conserved simple light response mechanism that mediates SE. Fortunately in Arabidopsis the light reception and response mechanisms are fairly well characterized, especially the red/far-red receptor family, PHYTOCHROME (PHY).

Red Light perception in *Arabidopsis thaliana*

Red and far-red light is received by the five member *PHY* (*A-E*) family in Arabidopsis except Wassilewskija (Ws) which lacks *PHYD*. As reviewed by Bae and Choi (2008) the PHY protein's major constituents (Fig L.5) are the N terminal PAS domain covalently linked to phytochromoblin and the C terminal protein interaction domains. Light activated nuclear import is mediated by binding of FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) for PHYA (Genoud *et al.*, 2008) and PHYTOCHROME INTERACTING FACTORs (PIF1, 3, 4, 5) for PHYB (Pfeiffer *et al.*, 2012). PIF3 is phosphorylated after light induced binding to PHY and then degraded by proteasomes. Shin *et al.* (2009) show that inhibition of *PIF1, 3, 4* and *5* is part of the mechanism by which seedlings respond to light through *PHY*. Phytochrome biological activity changes between its two conformations; the Pfr (far red absorbing) and Pr (red absorbing) forms.

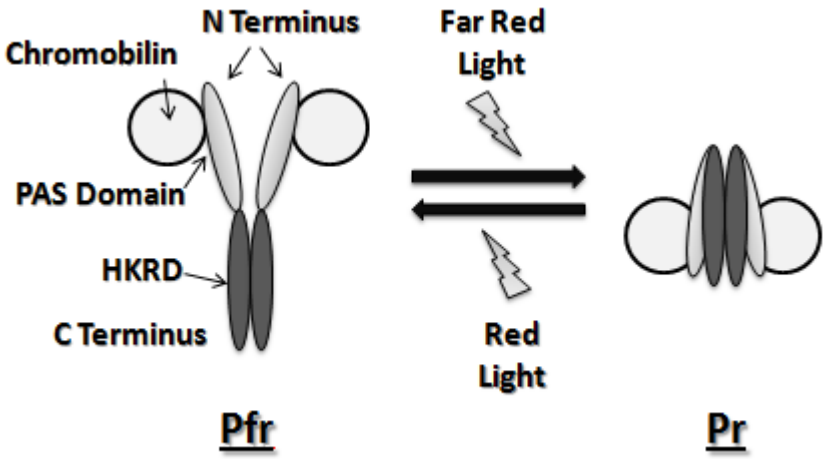

Fig L.5 Diagram of Basic Phytochrome Structure

The basic phytochrome structure consists of a light absorbing chromobilin protein covalently attached to the PAS domain at the N terminus and histidine kinase-related domain (HKRD) at the C terminus. Kinase activity is near the N terminus, while protein-protein interaction including dimerization is located at the C terminus. Phytochrome isomerizes between the Pfr (far red absorbing) and Pr (red aborbing) forms on light absorption. Adapted from Bae and Choi (2008).

Table L.1 Spectral Properties of Arabidopsis Phytochrome Proteins
Visible spectrum absorbance maxima of the Pr, Pfr isomers of PHY in Arabidopsis Col-0. Dark reversion of Pfr to Pr half life at 4°C *in vitro*.
Adapted from Eichenberg *et al* (2000)

	Pr (maxima)	**Pfr (maxima)**	**Dark Reversion (4°C)**
PHYA	651 nm	711 nm	30 min $_{50\%}$
PHYB	648 nm	709 nm	<6 min $_{50\%}$
PHYC	641 nm	701 nm	10 min $_{50\%}$
PHYD	N/A	N/A	N/A
PHYE	649 nm	700 nm	10 min $_{50\%}$

PHY isomerizes between P forms depending on the wave length of light absorbed (Table L.1). Under darkness, entropy favours shifting to the Pr conformation. As shown by Eichenburg *et al.* (2000) PHYs have different spectral activity and reversion kinetics. Clack *et al.* (2009) demonstrate that PHYs differ in dimerization characteristics as well. PHYA, PHYB and PHYD form homodimers, PHYB and PHYD can form a heterodimer while PHYC and PHYE can only dimerize with PHYB or PHYD. Their report also finds PHYE to likely have monomer activity as well as being the only PHY that does not bind to PIF3. The physical differences of the PHY molecules are likely the reason behind the difference in bioactivity.

PHY bioactivity appears to have low redundancy (reviewed by Franklin and Quail 2010). Mutant analysis has revealed *PHYA* to be involved in both high irradiance response and very low fluence light response, while *PHYB* handles low fluence response during germination, de-etiolation and gravitrophism. *PHYB*, *D* and *E* process different shade avoidance strategies, while *PHYA* seems to antagonize these responses. *PHYB* mutations also seem to affect stomatal index at high irradiance. *PHYA*, *B*, *D* and *E* can adjust circadian clock timing. All *PHY*s seem to be additively involved in flowering time.

*PHY*s also modulate stress responses (reviewed by Azevedo *et al.*, 2011). Salt stress studies suggest that salt tolerance protein negatively regulate PHYA and PHYB signals. PHYB signalling also is suggested to affect transpiration responding to drought stress. Temperature stresses, both cold and warm have also been linked to differential responses of *PHYA, B* and *D*. PHYs were also found to reduce jasmonic acid herbivory responses under far-red light conditions. Light dependent and light independent germination processes are controlled by *PHYA* and *PHYB* (Shinomura *et al.*, 1994) as well as *PHYE* (Hennig *et al.*, 2002).

Germination of *Arabidopsis thaliana*

Germination can be considered as two parts; disabling of growth arresting effects (dormancy) and resumption of growth (reviewed by Finch-Savage and Leubner-Metzger 2006). Dormancy can be further distinguished between shallow (seed coat-imposed) and deep dormancy where the embryo will not germinate under favourable conditions. The minimal conditions for germination are suitable temperature, oxygen and water.

Water imbibition of dry seeds is the first stage of germination (reviewed by Rajjou *et al.*, 2012). Imbibition reactivates metabolic activity and leads to the first check point for germination. If external conditions are unfavourable (season has not changed, temperature, shade and etc.) Arabidopsis seeds may actively promote dormancy. Dormancy and growth are predominately controlled by two phytohormones gibberellic acid (GA) and abscisic acid (ABA) (reviewed by Finch-Savage and Leubner-Metzger 2006).

ABA signalling promotes dormancy and arrests growth, while GA does the opposite (reviewed by Rajjou *et al.*, 2012). Increasing endogenous GA activity supersedes endosperm released ABA suppression (Lee *et al.*, 2012) only at the final steps after a seed commits to germinating. Committed seeds mobilize their fatty acid reserves through the peroxisomes via COMATOSE (CTS) to provide energy for rapid seed growth. From seed to seedling, almost all past research in Arabidopsis has focused on light germination and neglected the more agriculturally relevant dark germination.

Dark Germination of *Arabidopsis thaliana*

Dark germination in Arabidopsis has never previously been formally studied. In past literature dark germination has only been used as a characterization parameter in reference to light germination resulting in a lack of standardized testing conditions. Under such a background a review was crafted from results scrounged from experiments including dark germination in Arabidopsis.

Dark germination is affected by maternal light conditions. McCullough and Shropshire (1970) provided some early work suggesting that light conditions during maternal seed development affected dark germination response (up to 9 months later) in Estland. Their findings suggest that high ratios of far-red light to red light inhibited dark germination in the progeny while the high red to far-red was enhancing. Hayes and Klein (1974) tested the McCullough and Shropshire conclusions. They confirmed the findings with ecovar BL-1 in addition to Estland. Extending the work, they demonstrated that light effects required localization on the developing seed prior to desiccation. Since the dark germination effecting light was red and far-red it was not surprising that PHY was involved.

Shinomura *et al.*, (1994) observed that Landsberg (Ler) required functional *PHYB* to germinate in the dark. PHYB mediates proteosomal degradation of PIF1 (Shin *et al.*, 2009) and Oh *et al*. (2004) characterizing Col *pif1*, a basic helix-loop-helix (bHLH) protein, observed light indifferent germination. *PIF1* overexpression repressed germination response under darkness. The dark germination of *pif1* was not affected by PHYA loss of function, but reduced by PHYB loss of function.

PIF1 was found to directly enhance transcription of *SOMNUS (SOM)*, a CCCH-type zinc finger protein (Kim *et al.*, 2008). They obsereved that *som* germinated irrespective of light conditions. SOM increased ABA levels through changes in transcription of ABA metabolic genes. In addition they demonstrated that SOM down regulated *GA3ox1* and *GA3ox2* and up-regulated *GA2ox2* decreasing GA levels. Loss of function of *GA2ox2*, an enzyme that inactivates GA, resulted in increased dark germination in Col and Nossen (No) (Yukika *et al*., 2007).

GA3ox1 is also repressed in seeds by *DOF AFFECTING GERMINATION 1* (*DAG1*), a Dof family zing finger transcription factor (Gabriele *et al*., 2009). Loss of function *dag1* increased dark germination in Ws (Papi *et al*., 2000). Follow up work by Gualberti *et al.* (2002) reconfirmed the *dag1* results and found that the closely related *dag2* caused the opposite effect. Overexpression of *DAG1* resulted in a *dag2* like phenotype. Investigation of *DAG1* by Gabriele *et al.* (2009) found that PIF1 up-regulated *DAG1* expression resulting in reduced GA.

Debeaujon and Koornneef (2000) showed that Ler and Ws mutants for GA biosynthesis, *ga1*, could not germinate. Chemical inhibitors of GA biosynthesis could replicate the phenotype in the wild types, while exogenous GA rescued the mutants. Inhibition of ABA biosynthesis by norflurazon failed to rescue *ga1*. Testa mutants *ttg1* and *ttg4* allowed light independent germination, but only rescued *ga1* under light conditions.

The *ga1-3* mutant which does not germinate (Koornneef and Karssen 1994) was rescued by DELLA loss of function. Cao *et al.* (2005) working on DELLA family proteins, GA degraded GRAS transcription factors, in Ler discovered that triple or quadruple loss of DELLA (*rgl/rgl2/rga/gai*) function allowed light and GA indifferent germination. Of the DELLA proteins only *RGL2* was found to be up-regulated during imbibition (Lee *et al*., 2002).

RGL2 mediated release of ABA from the aleurone (endosperm) layer suppresses growth resumption (Lee *et al.*, 2010). GA mediated growth resumption is activated by PHYA (and potentially PHYE) in the embryo in response to continuous, non-red light, while endosperm PHYB mediates GA deactivation of ABA release by the aleurone (Lee *et al.*, 2012). Bethke *et al* (2007) demonstrated that the aleurone alone was sufficient and necessary for seed dormancy, as aleurone free embryos readily germinate including *ga1* (Debeaujon and Koornneef 2000). Their work also found the aleurone to maintain dormancy when treated with ABA or the NO scavenger c-PTIO, but was released by GA. Expression of *GA3ox,* the final step in bioactive GA synthesis, was only detected in the embryo.

Riefler *et al* (2006) revealed that loss of cytokinin (CK) receptors (AHK2/AHK3/CRE1) increased dark germination additively in Columbia (Col). This is odd as Wang *et al* (2011) found cytokinin to antagonize ABA based germination suppression by repressing *ABSCISIC ACID INSENSITIVE 5 (ABI5)* expression. ABI5 is a bZIP transcription factor that arrests growth in response to ABA downstream of ABI3 (Lopez-Molina *et al.*, 2002). ABI5 synthesis is promoted by low GA (not ABA), while ABA promotes the expression of the DELLA protein RGL2 which positively feedbacks to ABA biosynthesis (Piskurewicz *et al.*, 2008).

In summary (Fig L.6) red and far-red light during seed setting is “saved” into the seed at desiccation. The “save” effect likely acts to control germination through PHYB. Pfr PHYB promotes dark germination and also negatively regulates PIF1 (although it remains to be proven if Pr PHYB up-regulates PIF1). PIF1 maintains expression of SOM and DAG1, which maintain ABA and inhibit GA biosynthesis through GA3ox1 and GA3ox2. GA levels are actively repressed under darkness in part by GA2ox2 catabolism. GA degraded DELLA proteins represses germination in concert with ABA signalling as per the light germination derived model (reviewed by Finch-Savage and Leubner-Metzger 2006).

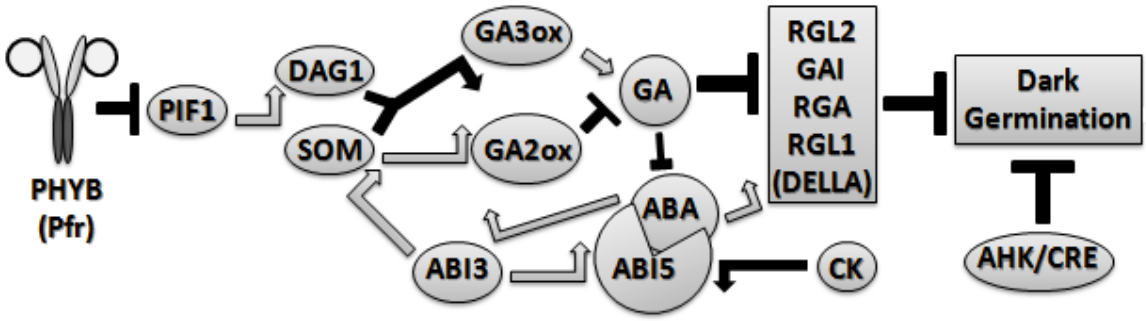

Fig L.6 Simplified Arabidopsis Dark Germination

This simplified diagram summarizes the dark germination molecular and genetic relationships in Arabidopsis. Bent arrows represent transcriptional changes, while block arrows denote activity suppression. Many details were omitted such as temporal-spatial, factors with unclear mechanisms and detailed molecular interaction (companion factors). PHYTOCHROME B (PHYB), PHYTOCHROME INTERACTING FACTOR 1 (PIF1), DOF AFFECTING GERMINATION 1 (DAG1), SOMNUS (SOM), ABA INSENSITIVE 3 & 5 (ABI3, 5), GIBBERELLIN 3-OXIDASE (GA3ox), GIBBERELLIN 2-OXIDASE (GA2ox), REPRESSOR OF GA (RGA), RGA-LIKE 1 & 2 (RGL1, 2), GA INSENSITIVE (GAI), HISTIDINE KINASE (AHK), CYTOKININ RESPONSE (CRE)

Previous work (above) has shown that germination components downstream of PHYB are well established. PHYB activation by red light leading to germination is known in detail (reviewed by Bae and Choi 2008); however dark germination precludes light mediated activation of PHYB. Thus upstream components of PHYB can be considered unknown. The work of this thesis examines components upstream of PHYB during dark germination.

Objectives

There were two objectives in this thesis, to elucidate the role of light during SE induction and to elucidate the cause of the *wox7* loss of dark germination phenotype.

Chapter 1 is a report on a project that could not progress further. This project attempted to examine the relation of *WOX* genes to SE. Due to problems encountered it was deemed unworkable, but the examination of the relationship of light to SE was salvaged from it along with the *wox7* dark germination phenotype.

In chapter 2 the effect of darkness and light quality on SE induction was investigated. The role of the *PHY* red light receptor family was also examined in relation to SE. Linking PHY light reception activity to known SE factors was the main objective of that chapter.

In chapter 3, experiments focused on examining dark germination in order to elucidate what causes loss of germination in *wox7*. Germination signaling factors were tested against *wox7* and these results were linked with the earliest factor known in dark germination, *PHYB*.

CHAPTER 1: SE PHENOTYPE OF *WOX* MUTANTS

1.1 Abstract

During somatic embryogenesis (SE) expression of *WUSCHEL* (*WUS*) is observed prior to embryo formation and ectopic expression of *WUS* induces SE. *WUSCHEL RELATED HOMEBOX* (*WOX*) genes share the same DNA-binding domain as WUS. Of the 14 *WOX* genes, 12 mutants (excluding *WOX2* and *WOX9*) were measured for SE response using an immature zygotic embryo system with 2,4-D as the inducer. The *wox8* and *wox12* mutants had reduced embryo production while the *wox7* and *wox13* mutants displayed enhancement. GUS expression of *WOX7* was transient during SE induction under light, while SE repressive dark induction had constitutive *WOX7* expression. The results suggest that *WOX7* is part of the SE repression machinery.

1.2 Introduction

Somatic embryogenesis (SE) is a plant specific phenomenon, where somatic cells undergo transition to embryogenesis resulting in a new plant. *In vitro* plant generation can help overcome recalcitrant or long breeding cycle species, or serve to maintain homozygosity in out-crossing species. *In vitro* embryogenesis is also a useful platform to investigate plant development and response (reviewed by Namasivayam 2007).

Development of SE resembles zygotic embryogenesis. During zygotic embryogenesis in *Arabidopsis thaliana*, Mayer *et al* (1998) found that the shoot apical meristem (SAM) transcription factor *WUSCHEL* (*WUS*) began expression in the SAM from the 16-cell stage onwards. Estradiol induced *WUS* expression during germination was found to cause embryo formation in Wassilewskija (Ws), Columbia (Col-0) and Landsberg (Ler) seedlings (Zuo *et al.*, 2002). They also showed that 35S::*WUS* expression halted SE development promoting shoot development instead, which has been replicated (Gallois *et al.*, 2004). *WUS* expression was observed to occur in embryogenic callus from immature zygotic embryo explants prior to SE (Su *et al.*, 2009). These results support a strong role for *WUS* in SE.

WUS was found to be a member of the larger *WUSCHEL RELATED HOMEOBOX* (*WOX*) gene family by Haecker *et al* (2004). Fourteen other *WOX* genes (*WOX1-14*) are found in Arabidopsis. Phylogenetic analysis (Fig 1.2.1) divides the *WOX* genes into 3 clades; the WUS clade (1-7), intermediate clade (8, 9, 11, 12) and ancient clade (10, 13, 14) (reviewed by van de Graff *et al*., 2009). *WOX* genes that have been phenotyped are all transiently expressed in tissue transitioning into another fate; i.e. *WUS* maintains SAM stem cell identity, while *WUS* free cells differentiate into shoot structures (Mayer *et al*., 1998). *WOX5* parallels this in the root apical meristem (RAM) with conserved mechanisms (Sarkar *et al*., 2005).

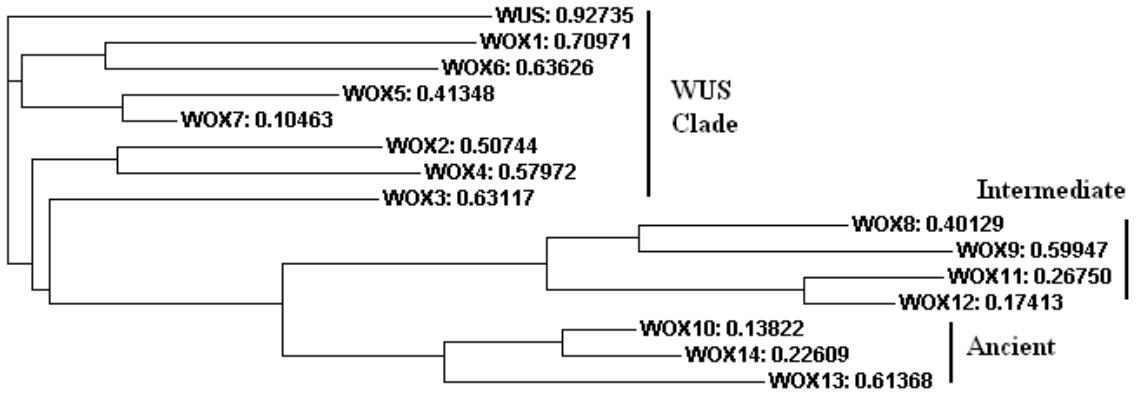

Fig 1.2.1 Phylogenetic relationship between WOX proteins in Arabidopsis
The 15 *WUS HOMEDOMAIN* (*WOX*) genes in *Arabidopsis thaliana* are divided into the WUS (*WUS* and *WOX1-7*), Intermediate (*WOX8, 9, 11, 12*) and Ancient clades (*WOX10, 13, 14*). Alignment and phylogenetic tree generated using Clustal (Sievers *et al*., 2011)

Lateral meristems also involve *WOX* function, with mutants displaying aberrant organ development (reviewed by van der Graff *et al*., 2009). Leaf bud meristems express *WOX3* and *WOX1* (Vandenbussche *et al*., 2009) while lateral root bud meristems express *WOX13* and *WOX14* (Deveaux *et al*., 2008). Retention of undifferentiated vascular meristems involves WOX4 activity (Hirakawa et al., 2010). *WOX* genes are involved in embryonic cell fate transition with *WOX6* regulating ovule development (Park *et al*., 2005), *WOX8/9* involved in zygotic basal differentiation and *WOX2* in apical differentiation (Wu *et al*., 2007).

While individual *WOX* genes contribute to varied developmental activity across the entire plant, they commonly share a few features (reviewed by van der Graff *et al.*, 2009). One of these would be that they seem to only alter activity in the cells which they are expressed in and descendent cells. Another is their transitory nature, the expression of *WOX* genes begins in "stem cells" and terminates where cells differentiate and mature.

Although only shown for *WUS* (Fletcher et al., 1999), *WOX5* (Stahl *et al.*, 2009) and *WOX4* (Hirakawa *et al.*, 2010), a feedback loop is used to terminate expression. *WOX* induced expression of an intercellular *CLAVATA* signaling peptide, *CLV3, CLE40* or *TDIF* is received by the respective *CLAVATA* receptor kinase, *CLV1, ACR4* or *TDR* to down-regulate *WOX* expression.

The WOX family has also been linked to photomorphogenesis through *WOX9*. Mutants of *wox9* were found to de-etiolate, a light promoted process, under darkness (Skylar *et al*., 2010). Light has been found to enhance SE induction in many species (reviewed by Gaj 2004). It is possible that the SE photo-induction effect may also act through a *WOX* family member. If it behaves like WOX9 then the light effect may work through cytokinin (CK).

Several type-A, CK induced negative feedback *ARABIDOPSIS RESPONSE REGULATOR*s (*ARR5, 6, 7, 15*) were observed to be repressed by *WUS* expression (Leibfried *et al*., 2005). They further demonstrated that constitutively active ARR7 mimicked the reduced meristem of *wus* mutants and conversely an increase in meristem size was observed with *arr7* mutants. WOX9 was observed by Skylar *et al.* (2010) to be required for SAM CK establishment and subsequent meristem stability, although WOX9 did not suppress type-A *ARR*s. Type-A *RR* expression in rice was found by to be suppressed by WOX11 during the establishment of crown root meristems (Zhao *et al.*, 2009). CK and auxin homeostasis are cross-regulated, and both hormones are involved in SE (reviewed by Gaj, 2004), as such it is likely that the effect of WUS on SE may be due to changes in CK patterning that affects auxin homeostasis.

As shown by Su *et al.* (2009), *WUS* expression is induced by exogenous auxin during SE. Auxin homeostasis *in planta* had been evidenced by work on *WOX5* and RAM auxin maxima establishment (Gonzali *et al.,* 2005), as well as work on embryonic auxin patterning by *WOX2* and *WOX8* (Breuninger *et al.*, 2008).

Exogenous auxin (2, 4-D) is the primary biochemical inducer of SE in this system. Raghavan (2004) illustrates the response of immature, bent-cotyledon stage zygotic apical cells to 2,4-D with SE arising from callus induced by auxin. Subsequent transfer to hormone free media is necessary for embryos to undergo maturation. Given that *WOX* genes affect auxin homeostasis and its crosstalk partner CK, it can be expected that some of the *WOX* genes are contributing components of the SE pathway(s). Knockdown mutants should hypothetically show either no difference or reduced SE competence, especially *WOX8* which is integral in zygotic embryogenesis development.

1.3 Results

Identification of wox Mutants Affecting Somatic Embryogenesis

Homozygous SALK (Alonso *et al.*, 2003) *wox* mutants (except 4 & 13, which were GABI-KAT & SAIL lines respectively) were screened for their SE response (Fig 1.3.1). Confirmation of WOX transcript levels were attempted using real time PCR, but failed due to the appearance of multiple peaks likely due to similarity between WOX members. Mutants could not be isolated for *WOX2* and *WOX9*. *WOX10* and *WOX14* are tandem inverted and share the same promoter region (~500 bp), in which the SALK T-DNA insert was located. The *wox8* and *wox12* had significantly lower SE response compared to the wild type (Fig 1.3.1).

Reduced somatic embryos formed per responding explant was the main contributing factor. The relative percentage of responding explants and somatic embryos formed per responding explant were lower in *wox12* compared to wild type. Oddly a marginal increase in total SE (Fig 1.3.1) was observed in *wox7* and a modest increase was seen in *wox13* (not pursued due to late discovery).

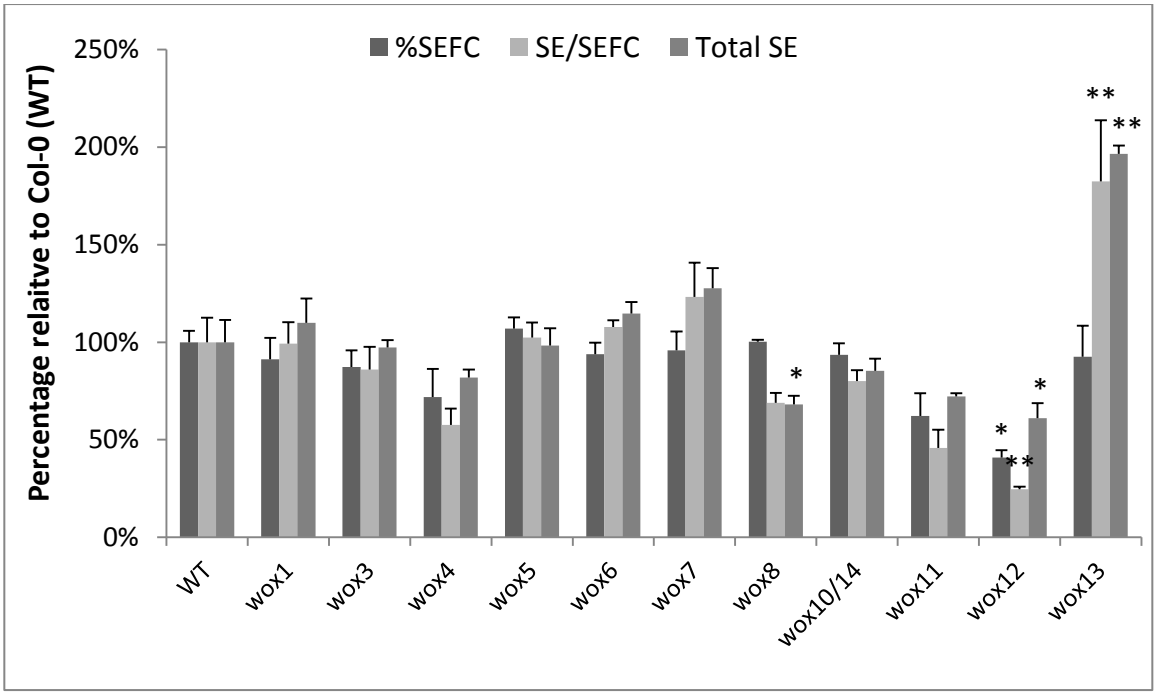

Fig 1.3.1 Somatic Embryogenesis Response of wox Mutants

The response of *wox* mutants to somatic embryogenesis of *Arabidopsis thaliana* Columbia. Immature, bent cotyledon zygotic explants were under light induction for 14 days followed by 7 days of light maturation. The percent of somatic embryo forming callus (%SEFC ± RSE), number of somatic embryos per SEFC (SE/SEFC ± RSE) and total somatic embryos per explants (Total SE ± RSE) was normalized to Col-0 (WT). Four replicates of 30 explants were used per line. Significance of difference from WT *: $p \leq 0.05$, **: $p \leq 0.001$ (One-way ANOVA). RSE = Relative Standard Error. Numbers available (Supplemental Table 1.A.1).

Table 1.2.1 Shortened list of cis-elements in WOX promoters

The -1000 bp upstream promoter sequences of all *WOX* genes for Arabidopsis Col-0 were obtained from The Arabidopsis Information Resource (TAIR). Sequences were parsed for cis-elements using the PLACE tool (Higo *et al.*, 1999). P-values from ATHENA (O'Connor *et al.*, 2005). Right column shows the *WOX* members whose promoters contain the element and the number of cis elements contained is shown in the brackets.

CIS Element	p-value	Function	*WOX* member (# of cis elements)
ACTTTA	**N/A**	**Auxin** induction	**1** (4), **2** (1), **3** (3), **4** (3), **6** (1), **7** (2), **8** (1), **9** (1), **10** (1), **14** (2)
NGATT	**N/A**	**ARR1 binding site**	**0** (9), **1** (11), **2** (8), **3** (9), **4** (9), **5** (12), **6** (17), **7** (18), **8** (15), **9** (11), **10** (6), **11** (10), **12** (14), **13** (12), **14** (14)
$C(A/T)_8G$	**0.0085**	**AGL15 binding site**	**All but WUS**
ACACNNG	**N/A**	**ABI5 binding site – ABA response**	**0** (2), **4** (1), **5** (6), **7** (1), **11** (1), **12** (2), **13** (1), **14** (1
GATAA	**0.7755**	**I-box – Light Response**	**1** (7), **2** (3), **3** (2), **4** (2), **5** (2), **6** (4), **7** (6), **8** (5), **9** (2), **10** (2), **11** (4), **12** (1), **13** (2), **14** (1)
AAMAATCT	**0.1178**	**CCA1 binding site – Phytochrome related**	**1** (1), **5** (1), **6** (2), **7** (1), **13** (1)

Analysis of Cis-Elements in the WOX Promoters

The promoter cis-elements in the 1000 base-pair upstream region were examined (Supplemental Tables 1.B.1, 1.B.2, 1.B.3) to narrow down the possible causes of SE differences in *wox* mutants. Biological significance, whether an element was known to affect SE, was considered more important than statistical significance. Some potentially interesting cis-elements have been highlighted (Table 1.2.1). The type-B ARR1 binding site was present with multiple copies on every *WOX* promoter. The multiple copies, ubiquitous distribution and known relation between *WUS*, *WOX9*, *WOX11* and CK suggest that perhaps all the *WOX* genes are involved in CK regulation. Apart from ARR1-binding motifs several *WOX* promoters had cis-elements related to the hormones (Supplemental Table 1.B.3) gibberellic acid (GA) and abscisic acid (ABA), which are important as their activities are required for SE maturation (Gaj *et al.*, 2006).

Another finding was AGAMOUS LIKE15 (AGL15) binding sites in all promoters except for *WUS* (Table 1.2.1). *AGAMOUS* (*AG*) is a negative feedback repressor of *WUS* (Lenhard et al., 2001) and *AGL15* is a necessary component of SE (Harding et al., 2003). Multiple light response elements were present in the *WOX7* promoter. Light as reviewed by Gaj (2004) plays an important but poorly understood role in SE.

wox7 Does Not Significantly Differ in Response to Light Induction of SE

To test the relationship between light, *WOX7* and SE, the difference between light and dark SE was measured (Fig 1.3.2). The results showed no significant difference between the SE response to light between *wox7* and wild type Col-0 under any combination of light and dark induction or maturation.

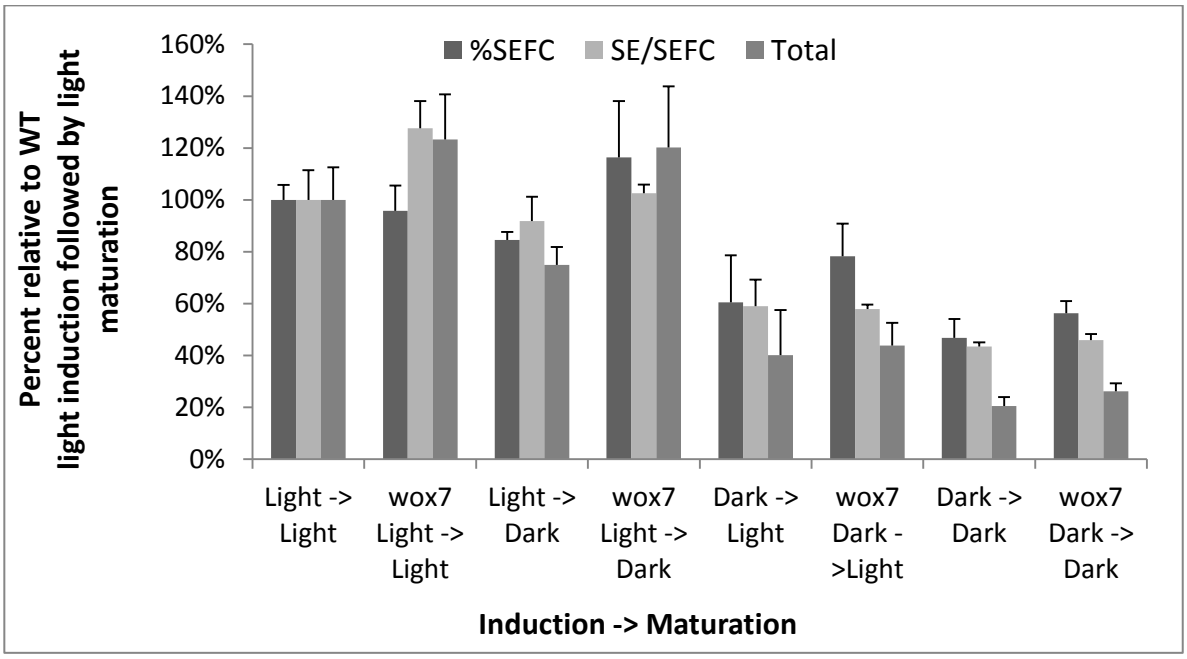

Fig 1.3.2 Somatic Embryogenesis Response of wox7 to Light

The effect of light on somatic embryogenesis of *Arabidopsis thaliana* Columbia wild type and wox7 mutant. Immature, zygotic bent-cotyledon explants were under induction for 14 days followed by 7 days of maturation. Induction light condition is listed on the left of -> and maturation light condition is listed on the right. The percent of somatic embryo forming callus (%SEFC ± RSE), number of somatic embryos per SEFC (SE/SEFC ± RSE) and total somatic embryos per explants (Total SE ± RSE) were normalized to the Columbia wild type under continuous light (Light -> Light). Four replicates of 30 explants were used per line. Significance of difference between WT and *wox7* of the same treatment *: $p \leq 0.05$, **: $p \leq 0.001$ (One-way ANOVA, pair comparison). RSE = Relative Standard Error Numbers available (Supplemental Table 1.C.1).

wox7 Does Not Respond Differently to GA, ABA, Auxin or Cytokinin

As the promoter analysis found cis-elements (Supplemental Table 1.B.3) for different hormonal signaling pathways, the hormonal response of *wox7* was examined. GA, ABA and CK prevented SE when added to media, so conventional hormone tests were used.

Compared to the Col-0 wild type, *wox7* did not have significant difference in the dark germination enhancement of GA (100% at 0.1, 1 and 10 µM GA_3) or the germination inhibition of ABA (Supplemental Figure 1.B.1). There was also no significant difference in auxin or CK inhibition of root growth (Supplemental Figure 1.B.2 & 1.B.3). No visual morphological difference between the wild type and *wox7* was observed during vegetative and floral growth or during the hormone response assays.

The *wox7* mutant also did not show any significant difference in transcript levels of SE markers *AGL15* and *LEC2* from the wild type at day 5 of SE (Supplemental Figure 1.C.1).

The Expression of WOX7 During Somatic Embryogenesis Induction

A homozygous SAIL (Sessions *et al.*, 2002) *wox7* mutant, which had pWOX7::GUS activity, was used to examine the expression of *WOX7* during SE. During the first few days of SE, *WOX7* was expressed, mainly at the sites of callus formation, under both light and dark induction (Fig 1.3.3, 1.3.4). GUS intensity was visibly stronger under darkness after day 4.

Callus formation occurred by day 5 regardless of light and continued to grow throughout induction. Organ like structures were not commonly formed during dark induction, whereas by day 8 under light apical-like protrusions could be found.

Deactivation of *WOX7* expression during light induction was observed starting at day 6 and ending after day 8 (Fig 1.3.3), whereas under darkness *WOX7* expression continued throughout induction (Fig 1.3.4). As the GUS protein is stable, the actual termination of expression may be a day or two before visible changes.

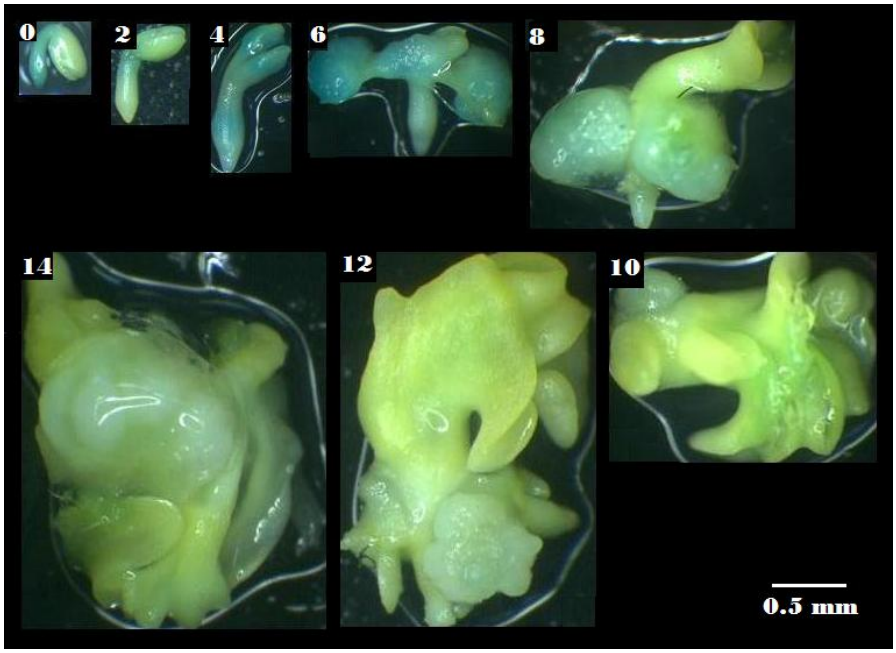

Fig 1.3.3 WOX7 Expression During Light Induction

Expression of pWOX7::GUS (SAIL) during somatic embryogenesis induction under 24 hours light. Starting from the pre-induction explant (0) then after 2 days (2), 4 days (4), 6 days (6), 8 days (8), 10 days (10), 12 days (12) and 14 days (14) on induction media. White scale bar indicates 0.5 mm.

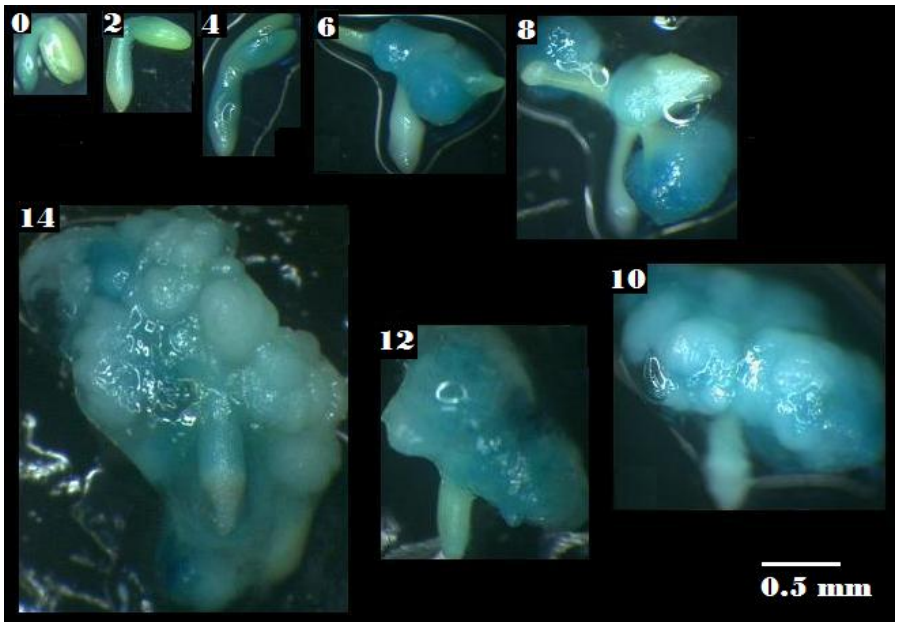

Fig 1.3.4 WOX7 Expression During Dark Induction

Expression of pWOX7::GUS (SAIL) during somatic embryogenesis induction under 24 hours darkness. Starting from the pre-induction explant (0) then after 2 days (2), 4 days (4), 6 days (6), 8 days (8), 10 days (10), 12 days (12) and 14 days (14) on induction media. White scale bar indicates 0.5 mm.

1.4 Discussion

The SE response of SALK (Alonso *et al.*, 2003) mutants was tested for 12 of the 14 *WOX* genes and *wox4*, *8*, *11* and *12* was found to have reduced SE competence (Fig 1.3.1). This was expected as *WUS* has been shown to be required for proper SE development (Zuo *et al.*, 2002, Su *et al*., 2009). It was not expected that the *wox7* and *wox13* mutants would have improved SE response. *WOX7* is an unidentified member of the WUS clade (reviewed by van der Graff *et al*., 2009), while *WOX13* is an ancient clade member, which is expressed in the lateral root bud primordia (Deveaux et al., 2008). With little literature available, *in silico* screening of promoter cis-elements was conducted in search for regulatory elements that may be related to SE.

Using the PLACE (Yoshida *et al.*, 1999) web-tool the known cis-elements present in the *WOX* promoters were examined. Elements biologically significant to SE were selected from the results, even when not statistically significant (Table 1.3.1).

Common to every *WOX* promoter were multiple copies of ARR1 binding sites (Table 1.3.1). ARR1 is a primary response factor of CK and CK (reviewed by Gaj, 2004, Gaj *et al.*, 2006) and the role of *WUS* (Leibfried *et al*., 2005), *WOX9* (Skylar *et al.*, 2009) and *WOX11* (Zhao *et al.*, 2009) in CK homeostasis has been demonstrated during shoot apical meristem maintenance, apical hook opening and crown root formation respectively. Cytokinin addition prevented SE in this system and so CK sensitivity could not be tested during SE. Hormonal response to CK was not altered in the *wox7* mutant during root inhibition tests (Supplemental 1.B.2). Thus it could not be concluded that *WOX7* was related to CK.

AGL15 binding sites were present in every *WOX* promoter except for *WUS* which has an AG binding site (Lenhard *et al*., 2001) with a probability due to random chance of 0.0085 relative to all Arabidopsis promoters (O'Connor *et al*., 2005). *AGL15* has been shown to be an important component of SE (Harding et al., 2003). The *AGL15* levels were not found to be significantly different between the wild type and *wox7* at day 5 of SE induction where *AGL15* transcription is increased and embryogenic callus development is usually first observable (Supplemental Figure 1.C.1). As such *AGL15* is

not likely to be a reason for the differences between *wox7* and the wild type. Moving on from the AGL15 binding site, testable cis-elements were examined.

Testable promoter elements included auxin, GA and ABA response elements common to several *WOX* promoters (Table 1.3.1). These major hormones are required for *in planta* development and Gaj *et al.*, (2006) observed that they were necessary for normal *in vitro* SE development. All three hormone response elements were found in *WOX7*'s promoter. Unfortunately GA and ABA prevented SE in this system and could not be tested during SE. There was no significant difference in hormone sensitivity to GA promotion of dark germination, ABA germination inhibition or auxin inhibition of root growth (Supplemental 1.B.3) between the Columbia wild type and the *wox7* mutant. Having found no significant difference in hormone response, the light motifs were investigated (Table 1.3.1).

The role of light in somatic embryogenesis is controversial, with some studies showing light induction and other showing light repression of SE (reviewed by Gaj et al., 2006). Testing the effect of light on induction and maturation, light was found to be a significant factor of SE response (Fig 1.3.2) as light induction was relatively higher then dark induction. The relative amount of responding callus and somatic embryos formed was not significantly different between the wild type and *wox7* under the same light conditions. However *wox7* produced relatively higher levels of somatic embryos under light induction, consistent with the previous trial (Fig 1.3.1). This suggests that the *wox7* phenotype was due to loss of function of inhibition, when light is present as a SE inducing signal; as opposed to gain of function in some SE competence mechanism since under darkness where SE response is lower, *wox7* relative total embryo levels are similar to the wild type. This would suggest that *WOX7* would normally be repressed under light conditions.

pWOX7::GUS from the SAIL lines (Sessions *et al.*, 2002) was used to observe the expression of *WOX7* during SE induction. Under light induction there was a clear expression of *WOX7* in the pre-callus followed by deactivation of *WOX7* expression in the developed callus (Fig 1.3.3), whereas there was continuous expression of *WOX7* under darkness (Fig 1.3.4). Given that light induction greatly enhances SE levels over

darkness (Fig 1.3.2) this suggests that *WOX7* is a peripheral component of the putative constitutive SE inhibition machinery that deactivates by light. The expression of *WOX13* during SE could not be examined due to a lack of a reporter line.

This study was able to establish some basic footwork in examining the relation between *WOX* genes and somatic embryogenesis. *WOX8* and *12* were identified as potential components in the process of SE, due to the significantly lowered SE levels in their mutants, although it will take future work to establish their actual roles. Surprisingly knockdown mutants of *WOX7* and *WOX13* improved SE. *WOX13* could not be examined in detail, but *WOX7* was found to be possibly a minor inhibitor and the inhibition mechanism it was part of was switched off by light during induction. *WOX* genes are likely to play some role in SE, but higher order mutants may be needed to overcome possible genetic redundancy.

1.5 Materials and Methods

Plant Material and Growth Conditions

All *Arabidopsis thaliana* plants used were Columbia. Wild types were Col-0. SAIL (Sessions *et al.*, 2002), SALK (Alonso *et al.*, 2003) T-DNA insertion lines (Col-3 and Col-0 backgrounds respectively) and other mutant lines were obtained from the Arabidopsis Biological Resource Center. Homozygous progenitors were screened by PCR (Supplemental Table 4.A). Primers and insertion locations were obtained from Signal Salk (http://signal.salk.edu/). Seeds were surface sterilized with 75% ethanol and 0.1% Triton-X for 10 min, followed by a 95% ethanol wash for 1 min. Seeds were dried on sterile filter paper before being sown on solid media (½Murashige Skroog (MS) [plantMedia], 1% sucrose, 0.3% Phytagel [Sigma®]). To synchronize the seeds they were stratified at 4^oC for 3 days. Plates were germinated under 24 h light (warm white fluorescent [Philips] at 50±5 umol s^{-1} m^{-2}) and kept for two weeks at 22 ± 2^oC. Seedlings were transferred into soil (Sunshine LA4 aggregate plus [Sungro®]) pots with individual plants separated by plastic dividers (Arasystem [Betatech]). Potted plants were grown in a growth chamber (16h/8h day night cycle, warm white fluorescent at 50±5 umol s^{-1} m^{-2}, $22^o/18^oC$) and watered with tap water twice a week.

DNA Extraction

Explants were homogenized in salt extraction buffer (1.25 mM EDTA, 40 mM TRIS, 12.5 mM NaCl, 0.025% SDS) and then mixed with chloroform. The mix was separated by centrifugation (15k g, 5 min) and the top aqueous layer was transferred. DNA was precipitated out by adding one volume of isopropanol and centrifugation (15k g, 5 min). Supernatant was discarded and the pellet was air dried. Dry pellets were dissolved in diethylpyrocarbonate (DEPC) treated water and boiled to evaporate residual isopropanol and denature any DNAse contaminants (96^{o}C, 10 mins) before storage at -20^{o}C.

Somatic Embryogenesis Culture Conditions

Green siliques were surface sterilized in 75% ethanol for 5 min. Immature zygotic embryos were obtained by hand dissection of embryo sacs inside the siliques. Late stage embryos (large green cotyledons) were preferred as mortality was higher in younger explants. Explants were kept for 14 days on induction media (Supplemental 4.B) and then transferred to hormone-free, solid media (½MS, 1% sucrose, 0.3% Phytagel [Sigma®]) to mature for 7 days. Induction was under 24 h light (warm white fluorescent at 50±5 umol s^{-1} m^{-2}) and maturation was under 24 h darkness both at 22±2^{o}C. Somatic embryos were scored based on the following criteria: Identifiable, distinct apical and basal regions; independence of epidermis and vascular system from the original explant or other new formed somatic structures. Scoring was done by physically separating every potential embryo by hand and inspection under microscope. Each experiment was trialed once.

Promoter Cis-Element Analysis

The -1000 base pair upstream promoter sequences of all *WOX* genes and *WUS* for Arabidopsis Columbia were obtained from The Arabidopsis Information Resource (TAIR). Sequences were parsed for cis-elements using the PLACE tool (Higo *et al.*, 1999) & ATHENA provided p-values for cis-elements when possible (O'Connor *et al.*, 2005).

P-value described on the ATHENA website: (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/Tutorial_Statistics.pl).

RNA Extraction

RNA extractions were modified from the LiCl method described by Onate-Sanchex and Vicente Carbajosa (2008). Polyvinylpyrrolidone-40 (4% $_{w/v}$) was added to the extraction buffer (0.4 M LiCl, 0.2M TRIS, 25mM EDTA, 1% SDS) to protect against phenolics and reduce phenol contamination. Homogenized samples were phase separated by 1:1 volume chloroform and centrifugation (15k g, 5 min). The aqueous layer was re-separated by 1:1 volume of phenol and 2/5 volume of chloroform and centrifugation (15k g, 5 min). RNA was precipitated overnight (-20^{o}C) by increasing LiCl concentration to 2 M. After thawing the solution the RNA was pelleted by centrifugation (15k g, 30 min). After discarding the supernatant the pellet was then dissolved in 500 µl DEPC treated water. Carbohydrates were removed by precipitation (7 µl Na acetate, 250 µl EtOH) with centrifugation (15k g, 10 min). Supernatant was transferred and RNA was ethanol precipitated (50 µl Na acetate, 500 µl EtOH) at -20^{o}C for one hour. RNA was pelleted by centrifugation (15k g, 30 min). After discarding the supernatant the pellet was dissolved in 20 µl DEPC treated water. RNA extraction for non-seed explants skipped the first chloroform separation step. RNA concentration and quality was determined by nanodrop [Biorad®] after dissolving of recovered RNA in DEPC treated water. Good quality was a value ≥ 2 for the 260/230 ratio and ≥ 1.8 for the 260/280 ratio. DNA was removed from samples using 10 units of DNase I recombinant (Roche®) at 37^{o}C for 1 hour. Dnase was inactivated by boiling (96^{o}C, 15 min) RNA samples were stored at -80^{o}C.

PCR and RT-qPCR

PCR was carried out using a (1:2:6:6) mixture of DNA template, primer, water and BioMix [Bioline®]. Confirmation reactions for SALK lines were run using the following cycle; 94^{0}C (5 min) {94^{o}C (30 s): 60^{o}C (30s): 72^{o}C (45s)} x36 cycles. SAIL & GABI-KAT lines were run with a 56^{o}C annealing temperature. Reverse transcriptase reactions were performed following the Reverse Transcription System (Promega®, A3500) protocol for 2 µg of RNA using random hexamers (1 h at 37^{o}C followed by 5 min at 85^{o}C). RT-qPCR was done on a CFX96 (Biorad) with Sso-fast™ Evagreen® (Biorad) using the following cycle; 95^{o}C (2 min), {95^{o}C (3s): 60^{o}C (7s)} x39 cycles. Following the protocol from Biorad each reaction was an 8 µl mix consisting of 4 µl of Evagreen, 1

µl of primer mix and 3 µl of cDNA. All primers were designed to have a Tm at 60^{o}C (Supplemental Table 4.A.2).

Real Time Analysis

All samples were pooled from 150 (day 3 SE induction) or 50 (day 5 SE induction) individuals. Each experiment had three biological replicates. Cycle threshold (Ct) values were taken as the average of three technical replicates and outliers were thrown. All Ct values were normalized to a housekeeping gene (*UBQ10*) using the ΔΔCT method and corrected for primer efficiency (Livak and Schmittgen 2001). Primer efficiencies were calculated from the slope of four 4x serial dilutions for each gene.

Hormone Response Assays

Hormone response assays were modified from Weigel and Glazebrook (2002). All hormones were dissolved in 95% ethanol and applied after auto-claving, with controls receiving an equal amount of ethanol. Each experiment was trialed once. Auxin and Cytokinin inhibition of root growth was assayed by transferring 7 day old seedlings to vertical ½ MS agar plates with 1% sucrose. Plates were kept in the dark at 22±2^{o}C. Five wild type and five *wox7* seedlings were placed in alternation on a plate. 4 replicate plates were used. Auxin was applied as IAA [Sigma®] at 0.1, 0.5, 1, 5 and 10 µM. Cytokinin was applied as 2IP [Sigma®] at 0.001, 0.01, 0.1, 1, 5 and 10 µM. ABA germination inhibition was assayed by stratifying 3 replicates of 50 seeds on ½ MS + 1% sucrose agar plates for 3 days at 4^{o}C. Plates were then transferred to 24 hours light at 22±2^{o}C. Germination percentages were scored 7 days after transfer. ABA [Sigma®] was applied at 1, 5, 10, 20, 40 µM. GA dark germination was assayed using 3 replicates of 50 seeds on ½ MS + 1% sucrose agar plates. Plates were kept in the dark at 22±2^{o}C. Germination percentage was scored 7 days after sowing. GA3 [Sigma®] was applied at 0.1, 1 and 10 µM.

Microscopy and GUS Staining

GUS staining was done as described previously (Weigel and Glazebrook 2002). Explants were fixed with 90% acetone for 15 min at 4^{o}C. Explants were then incubated with GUS staining solution (1 mM EDTA, 5 mM $KFe(CN)_6^{-4}$, 5 mM $KFe(CN)_6^{-3}$, 100 mM $NaPO_4$, 1% Triton-X, 2 mM X-Gluc) at 37^{o}C for 4 hours, except pWOX7 detection during dark germination which was 24 hours. Microscope pictures were taken with a 420 DFC [Leica] attached to a Wild M8 dissecting microscope [Leica].

Statistical analysis

All statistical analysis was performed with one-way ANOVA tests. Somatic embryogenesis data was tested after normalization to the control. Real-time PCR data was tested after ΔΔCT (Livak and Schmittgen 2001) normalization and before logarithmic transformation. Other data was tested without further manipulation. SigmaPlot© [Systat®] was used for calculations.

CHAPTER 2: LIGHT INDUCTION OF SE

2.1 Abstract

The effect of light on somatic embryogenesis (SE) has been examined in many species; however no mechanism of action has been elucidated. Using Arabidopsis Columbia immature zygotic explants the inductive effect of red light (660 nm) was examined. Red light signaling resulting in SE was found to be mediated by PHYE based on *phyE* SE response. Both *phyE* and darkness resulted in several hundred fold lowered *CONSTITUTIVE PHOTOMORPHIC DWARF 3 (CPD)* transcription, which is a rate limiting step in BR biosynthesis, as well as *AGAMOUS LIKE 15* (*AGL15*), a key inducer of many SE genes. Application of BR to phyE and dark induced wild type partially restored *AGL15* expression. The results suggest that SE is induced by red light signaling through PHYE followed by BR signaling leading to AGL15 activity.

2.2 Introduction

The ability to generate embryos from somatic cells is a plant specific phenomenon that has become increasingly attractive. Commercially, it is being explored as a means of vegetative propagation. For research, it provides an *in vitro* method of studying embryogenesis and as a means of genetic transformation in some species (reviewed by Vicient and Martínez, 1998). Of course, deciphering the mechanisms leading to somatic embryogenesis (SE) is also interesting.

SE induction, the mechanism by which competent cells become determined to form somatic embryos, is one of the most important and yet "poorly understood" aspects of SE. Somatic embryogenesis induction is the response of explants to *in vitro* culture conditions to form somatic embryos (reviewed by Gaj, 2004). Arabidopsis immature zygotic embryos can be induced to undergo SE by auxin, with subsequent culturing on hormone free media to allow embryos to mature (reviewed by Gaj, 2004). Induction of SE without auxin has been reported to occur with application of stresses such as heavy metals or high osmolality (reviewed by Zavattierri *et al.*, 2010). While minimal induction

provides a basal level of SE, other factors affect the total induction of SE, one of these being light signaling.

Light effects on induction in Arabidopsis SE are not well studied. Gaj (2002) found that light inhibited the frequency of explants developing embryos from early stage zygotic explants and no significant difference versus darkness for late stage embryo explants. However Gaj's study did not measure the embryogenic capacity of individual explants. Apart from Arabidopsis the effect of light signaling on SE has been investigated in a few species such as carrot (Michler and Lineberger, 1987) and quince (D'Onofrio *et al.*, 1998). While the systems used differed (cotyledon cell suspension for carrots and leaves for quince) both results agreed that induction was found to be inhibited by high intensity blue light, and red light was found to enhance SE.

Red light is perceived through phytochromes. As reviewed by Bae and Choi (2008), Arabidopsis (except for WS) possesses 5 phytochromes (PHYA to PHYE). PHY isomerizes between the far red absorbing Pfr isomer and red absorbing Pr isomer upon light absorption and in PHYB this was found to be also modulated by ARABIDOPSIS RESPONSE REGULATOR 4 (ARR4) activity (Sweere *et al.*, 2001). The 5 PHYs are only loosely related (Clack *et al*., 1994) with PHYE sharing only about half amino acid identity with other PHYs. Though all PHYs bind to the same light-absorbing chromaphores, they have different spectral absorbance and dark revision kinetics (Eichenberg et al 2000). Furthermore, though all PHYs can dimerize, PHYC and PHYE form obligate heterodimers with PHYB and PHYD (Clack *et al*., 2009). Interestingly they found that PHYE did not complex with PHYTOCHROME INTERACTING FACTOR 3 (PIF3), which others have attributed to nuclear translocation and transcriptional changes (Pfeiffer *et al.*, 2012).

Phytochrome signaling can affect transcription, such as when red light up-regulates *CONSTITUTIVE PHOTOMORPHIC DWARF 3* (*CPD*) as observed by Bancos *et al* (2006). *CPD* is a rate limiting step in the brassinosteroid (BR) synthesis pathway (Szekeres *et al.*, 1996). BR application has shown positive enhancement in some SE systems such as cotton (Aydin *et al.*, 2006), coconut (Azpeitia *et al*., 2003) and canola (Belmonte *et al*., 2010), while BR antagonists down-regulated canola microspore derived

SE (Belmonte *et al*., 2010). As the name suggests, BR mutants were first identified by defective photomorphism. Constitutive photomorphism in the dark like *cpd* was also observed in the first identified BR receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Wang *et al*., 2001).

BRI1 is interesting because it has been shown to dimerize with SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 (SERK1) (Kalova *et al.*, 2006). SERK1 is a leucine-rich repeat containing receptor-like kinase (LRR-RLK), first identified as a SE marker in carrot (Schmidt *et al*., 1997). Overexpression of *AtSERK1* increased SE in Arabidopsis (Hecht *et al*., 2001). SE is also enhanced by up-regulation of the MADS-Domain transcription factor *AGAMOUS-LIKE15* (*AGL15*) (Harding *et al*., 2003), which Karlova *et al.*, (2006) demonstrated to interact with *SERK1*. SERKs 1 to 4 of the five member family in Arabidopsis were observed to co-mediate BR signaling mediated by BRI1 (*Gou et al.*, 2012).

A hypothesis can be formed where red light induction of SE is perceived by phytochromes and transduced through BR signaling to SERK and ultimately leading to AGL15 based transcriptional changes (Fig 2.2.1). Light signaling in Arabidopsis SE induction has not been elucidated; this chapter attempts to test the model using late stage zygotic embryo explants. First a difference in response to light and quality is expected, with red light being most inductive. Second one or more *phy* mutants from the SALK lines (Alonso *et al.*, 2003), if the PHYs are not redundant, should exhibit significantly reduced SE competence. This should be followed by light enhanced BR synthesis. Since BRI1 is the only BR receptor shown to dimerize with SERK1 at this time, *bri1* is expected to show reduced SE if BRI1 activity is non redundant. *serk1* and *agl15* have been shown to have decreased SE competence in other Arabidopsis SE systems and these results are expected to be replicable.

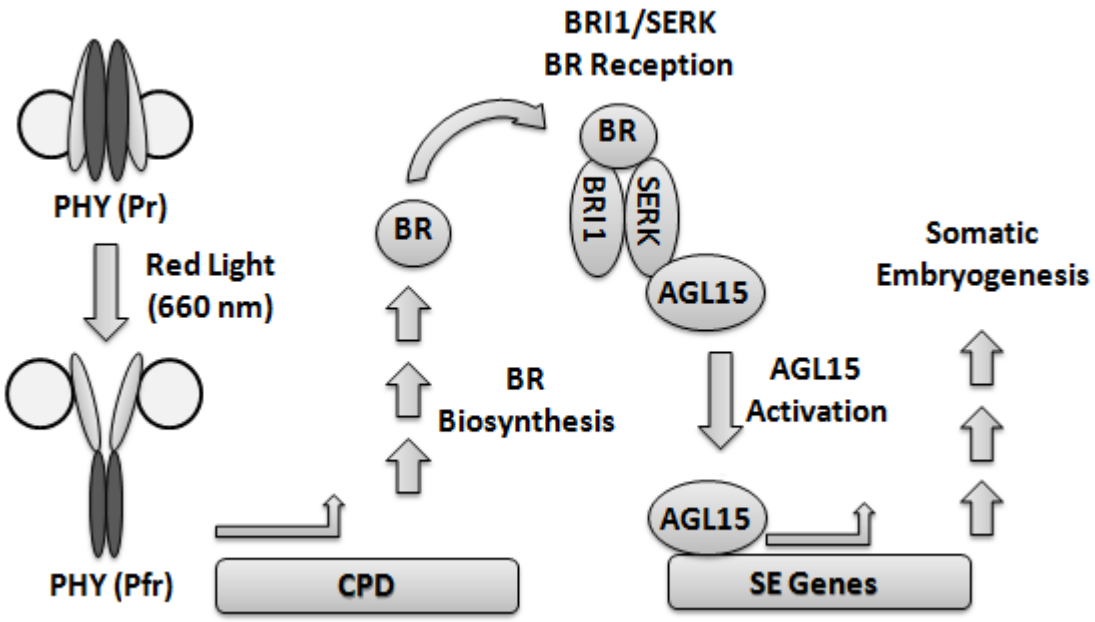

Fig 2.2.1 Hypothetical Model of Phytochrome Activation Leading to Somatic Embryogenesis

PHY in the red absorbing form (Pr) is isomerized into the far red absorbing form (Pfr) by red light. Pfr increases transcription of BR biosynthesis enzymes such as *CPD* during SE induction. Brassinosteroids activate BRI1/SERK complexes, which activate AGL15. AGL15 promotes SE related transcription activity, ultimately leading to SE. PHYTOCHROME (PHY), CONSTITIUTIVE PHOTOMORPHOGENIC DWARF (CPD), BR INSENSITIVE 1 (BRI1), SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK), AGAMOUS LIKE 15 (AGL15)

2.3 Results

Light Quality Controls Level of Somatic Embryogenesis Response

First the effect of light on the induction of SE in this system of SE was verified. All four possible pairings of continuous white light or darkness treatments during induction and maturation were examined (Fig 2.3.1). Darkness markedly reduced SE formation during induction and slightly during maturation. Light induction followed by light maturation produced the highest number of embryos compared to other treatments. Light maturation in all cases slightly increased SE. Since the goal was to examine induction specific effects of light, dark maturation was used for all following experiments to remove the change in SE due to light in maturation.

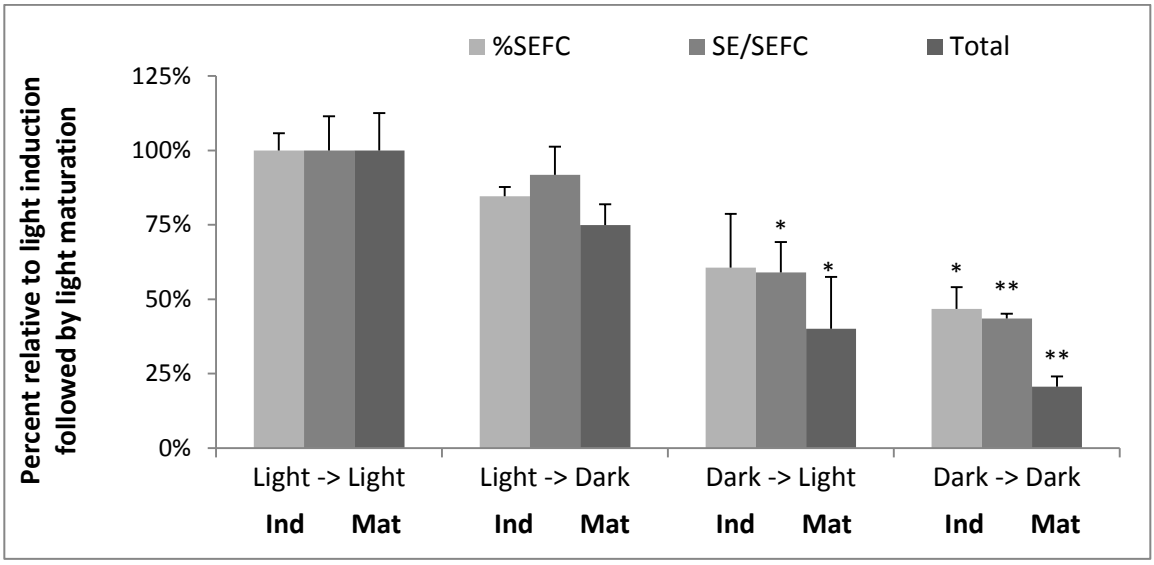

Fig 2.3.1 Somatic Embryogenesis Response to Light and Darkness

The effect of continuous white light or darkness during somatic embryogenesis induction (Ind) and maturation (Mat) of *Arabidopsis thaliana* Col-0 relative to continuous white light treatment. Percentage of callus forming somatic embryos (%SEFC ± RSE), somatic embryos per SE forming callus (SE/SEFC ± RSE) and total embryos per explant (Total ± RSE) were measured from 30 explants with 4 replicates. Significance of difference from (Light -> Light) *: $p \leq 0.05$, **: $p \leq 0.001$. (One-way ANOVA). RSE = Relative standard error. Numbers available (Supplemental Table 1.C.1).

The components of the visible spectrum known to have biological activity, far red, red, blue and green were tested for SE induction (Fig 2.3.2). Only red light had no significant difference from white light treatment. Other light treatments were grouped with darkness, but far red and green light also grouped with white and red. Far red and green light during induction increased the relative amount of somatic embryo forming callus and total somatic embryos compared to darkness, while total somatic embryos were relatively reduced compared to white light. Blue light was similar to darkness, but had higher variance. A few explants under blue light were able to reach white light levels, suggesting that under very specific conditions, likely due to the internal state of the initial explant, blue light can activate SE. Overall SE response was lower in this particular trial, which is expected as variance during *in vitro* morphogenesis is high.

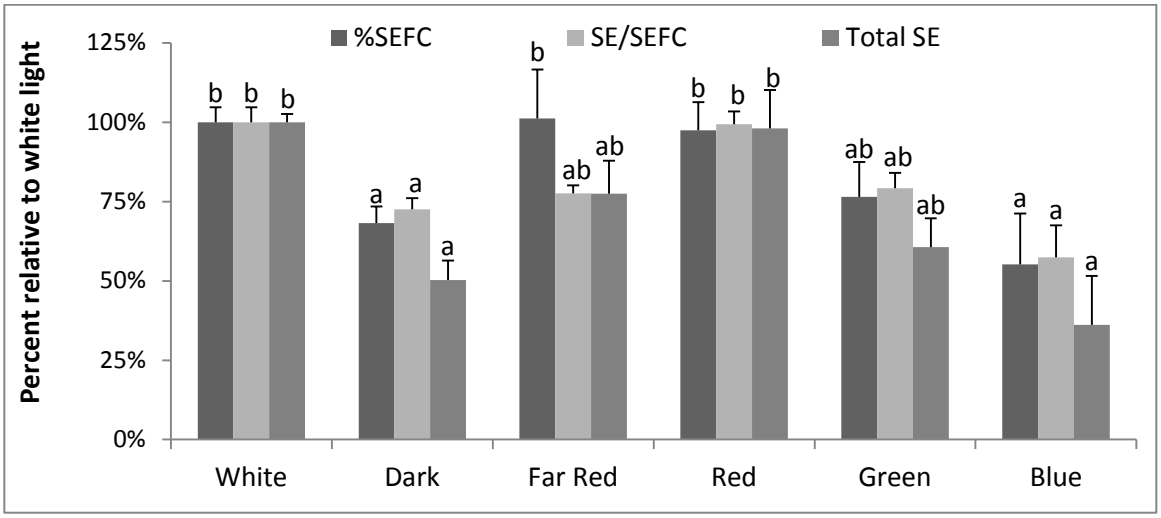

Fig 2.3.2 Somatic Embryogenesis Response to Light Quality

The effect of light quality during somatic embryogenesis induction of *Arabidopsis thaliana* Col-0 relative to continuous white light induction (White). All explants were matured in complete darkness. Far Red 740 nm; Red 660 nm; Green 540 nm; Blue 470 nm. Percentage of callus forming somatic embryos (%SEFC ± RSE), somatic embryos per SE forming callus (SE/SEFC ± RSE) and total embryos per explant (Total ± RSE) were measured from 30 explants with 4 replicates and normalized to White. Statistical significance between groups; $p \leq 0.05$ (One-way ANOVA, pairwise). RSE = Relative standard error. Numbers available (Supplemental Table 2.A.1).

PHYE Mediates Red Light Induction of Somatic Embryogenesis

Having verified that red light is the principle inducer of SE in Arabidopsis, the next step was to examine red light receptor mutants. SALK mutants for *phyA*, *phyB*, *phyD* and *phyE* were obtained; no *phyC* mutant could be isolated. Mutants of phytochrome A and B were not significantly different from wild type SE competence (Fig 2.3.3). The SE response of *phyD* mutants showed enhanced relative amount of callus forming embryos. The *phyE* had significantly reduced relative number of embryos formed per callus forming explants. Due to the higher variance in the total embryos formed by the wild type, the significant differences in SE response components in *phyD* and *phyE* did not result in significant differences in relative total embryos.

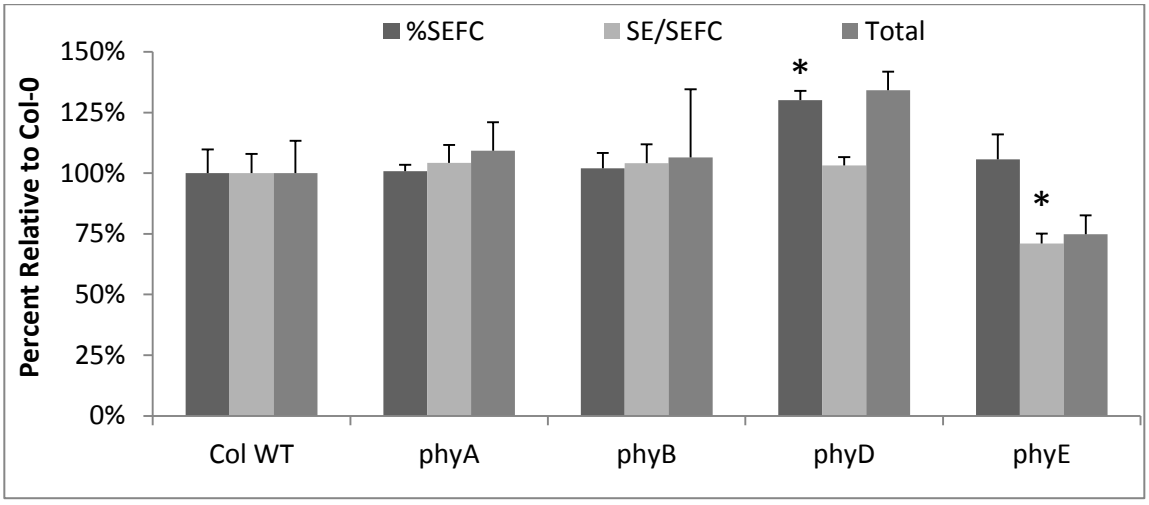

Fig 2.3.3 Somatic Embryogenesis Response of phy Mutants
The somatic embryogenesis competence of Arabidopsis phytochrome (*phy*) mutants during somatic embryogenesis relative to the Col-0 wild type. Percentage of callus forming somatic embryos (%SEFC ± RSE), somatic embryos per SE forming callus (SE/SEFC ± RSE) and total embryos per explant (Total ± RSE) were measured from 30 explants with 4 replicates and normalized to the WT. Significance of difference from Col-0 WT *: $p \leq 0.05$, **: $p \leq 0.001$ (One-way ANOVA). RSE = Relative standard error. Numbers available (Supplemental Table 2.B.1).

CPD, ARR4 and IPT7 are Induced by Red Light During Somatic Embryogenesis

Having confirmed darkness and *phyE* as antagonistic to SE in terms of embryos formed per responding callus, the early mRNA changes were compared to the Columbia wild type. The initial reference point was chosen as 3 days in induction; explants could be visibly confirmed to have survived by apical de-hooking and size increase. The point of comparison was chosen as day 5; callus formation is typically visible on this day. Callus formation occurs under both darkness and light (Fig 1.3.3, Fig 1.3.4), so transcript differences between light and darkness are likely to be specific to SE.

CPD, AGL15, and *ARR4* transcripts were strongly up-regulated at day 5 for the wild type under light, but not under darkness or in the *phyE* mutant (Fig 2.3.4). Furthermore darkness and *phyE* mutants did not have significant levels of *LEC2* (Supplemental Figure 2.A.1), which was below detection at day 3, but present at day 5 under light. *ARR4* levels were increased in all treatments at day 5, but only around 10 fold for darkness or *phyE* compared to almost 1000 fold under light (Fig 2.3.4). *AGL15* and *CPD* expression was increased several hundred folds from day 3 at day 5 under light induction. Both transcripts did not significantly increase under darkness or in *phyE*. The increase in mRNA levels is likely due to increase in expression area in addition to cellular transcript levels, as the RNA was extracted from the whole explant. Another possibility is increased response at the population level, as 50 samples were pooled together per replicate.

Since CPD is a rate limiting step for BR biosynthesis and both darkness and *phyE* had several hundred fold reduced *CPD* transcripts the effect of exogenous brassinosteroid under darkness in the wild type and for *phyE*, 24-epibrassinolide (EBL) was examined. Application of 2 µM of EBL partially restored *AGL15* expression by about a 100-fold for both darkness and *phyE* (Fig 2.3.4). *LEC2* transcripts were slightly increased by EBL treatment (Supplemental Figure 2.A.1). *CPD* and *ARR4* were not significantly affected by EBL. The results suggest that BR is involved in the up-regulation of *AGL15*.

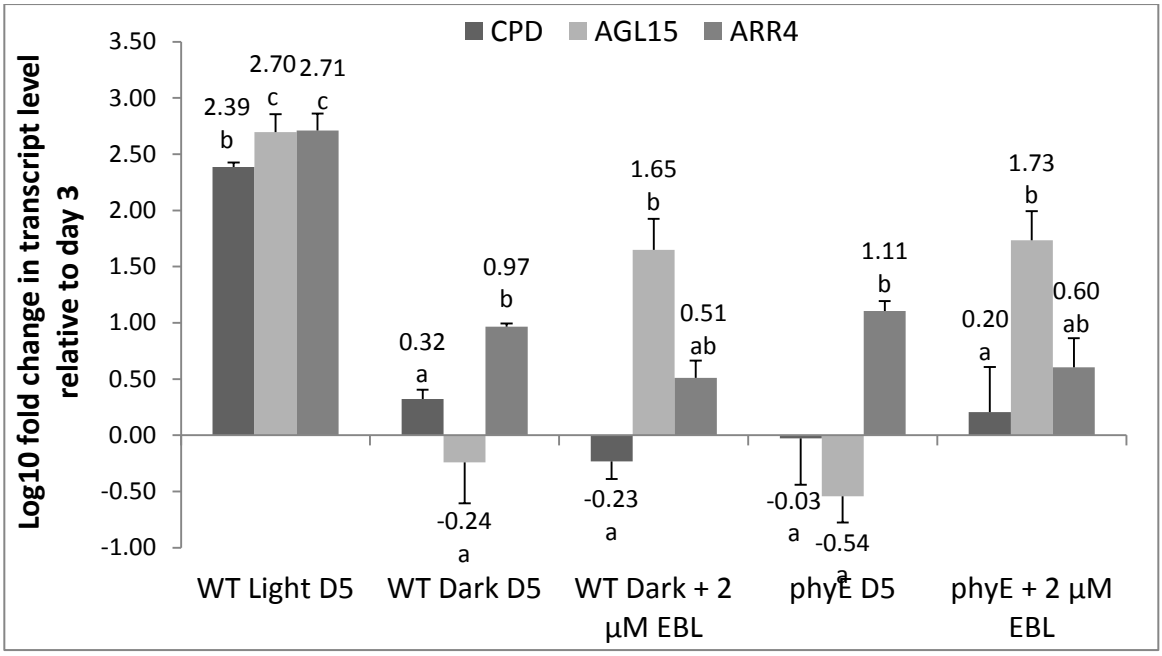

Fig 2.3.4 Relative Gene Expression Between Day 3 and Day 5 of SE Induction
Relative gene expression of *CONSTITUTIVE PHOTOMORPHOGENIC DWARF* (*CPD), AGAMOUS LIKE 15* (*AGL15)* and *ARABIDOPSIS RESPONSE REGULATOR 4* (*ARR4)* between day 3 and day 5 of somatic embryogenesis induction. *CPD*, *AGL15* and *ARR4* transcript levels ± Standard Deviation (SD) from day 5 treatments (Col-0 WT 24 h light, Col-0 WT 24 h dark, Col WT 24 h dark + 2 µM EBL, *phyE* 24 h light and *phyE* 24 h light + 2 µM EBL) were divided by day 3 levels under continous light. RNA was collected from 50 explants on day 5 and day 3 with 3 biological replicates. Statistical significance between groups; $p \leq 0.003$. Group a is not significantly different from day 3 wild type. (One-way ANOVA, pairwise)

SERK1, SERK5 and AGL15, but not BRI1 mediates somatic embryogenesis

Highly increased *CPD* mRNA levels suggest that BR biosynthesis may be up-regulated during SE. So the BR receptor mutant *bri1*, which displayed the cabbage like vegetative phenotype and high male sterility (Clouse *et al*., 1996), was tested for SE response. No significant difference between the wild type and *bri1* or BR downstream component *bes1* was observed for SE (Fig 2.3.5). The BRI1 associated SERK1 and its interacting partner AGL15 were reconfirmed to lower SE responses in mutant lines. Lowered SE was also observed in the *serk5* mutant, while *serk3* enhanced SE. Both *serk1* and *serk5* had lowered embryo formation, but the relative amount of callus forming embryos was not significantly different from wild type. The *serk2* and *serk4* mutants had no significant effect on SE.

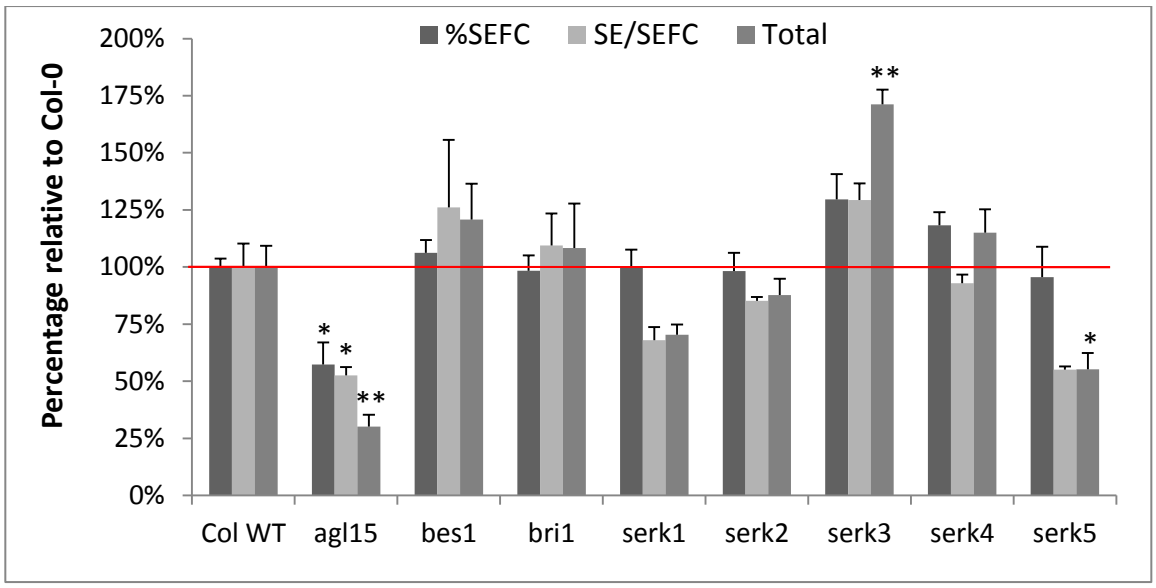

Fig 2.3.5 Somatic Embryogenesis Response of agl15, bes1 and serk Mutants
The effect of *agl15*, *bes1*, *bri1* or *serk1-5* mutation during somatic embryogenesis in *Arabidopsis thaliana* relative to Columbia wildtype. Percentage of explants forming somatic embryos (%SEFC ± RSE), somatic embryos per SE forming explants (SE/SEFC ± RSE) and total embryos per explant (Total ± RSE) were measured from 30 explants with 4 replicates normalized to the WT. Significance of difference from Col-0 WT *: $p \leq 0.05$, **: $p \leq 0.001$ (One-way ANOVA).RSE = Relative standard error. Numbers available (Supplemental Table 2.C.1). *AGAMOUS LIKE 15 (AGL15), BRI1 EMS SUPPRESSOR 1 (BES1), BR INSENSITIVE 1 (BRI1), SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE (SERK1-5)*

2.4 Discussion

A hypothetical model for light induction of somatic embryogenesis (Fig 2.2.1) was tested using an Arabidopsis immature zygotic SE system. The results suggest that light is an enhancer of late stage SE induction (Fig 2.3.1) in contrast to the lack of significant difference found by Gaj (2002). Investigating the effect of light quality in the visible spectrum, the results agreed with Michler and Lineberger (1987) as well as D'Onofrio *et al* (1998) in that red light was the principle inducer and blue light was antagonistic (Fig 2.3.2). Careful inspection however suggests that blue light can stimulate specific explants, which may explain why Gaj's review (2004) displays contradictory findings. Possible reasons may be the stage of the explants, or different abiotic conditions during zygotic embryo development such as shading, which would shift the light environment. Red light induction was stable and red light signaling was well established so investigation of phytochrome mutants was conducted.

Phytochrome E was found to transduce the red light signal to the SE pathway (Fig 2.3.3). Unfortunately as a *phyC* mutant was not obtained, it could not be proven as the sole receptor responsible. *PHYE* however does provide some explanations of the other results. While *PHYA* (reviewed by Bae and Choi, 2008) controls many far-red responses, *PHYE* has been found to partially mediate continuous blue and far-red light induced germination (Hennig *et al*., 2002). Far-red light induction produced a better than darkness result for SE (Fig 2.3.2), which may be due to PHYA or PHYE activity. SE had higher variance in *phyB* and had higher competence in *phyD* mutants (Fig 2.3.3). PHYE can only form dimers with PHYB and PHYD (Clack *et al*., 2009). This suggests the possibility that PHYB/E dimers may have better SE activity; further investigation would require *phyB* or *phyD* plus *phyE* double mutants to examine epistasis. Monomer activity has not been formally shown in literature, but *phyB*/*phyD* double mutants could see whether PHYE monomers have activity.

Darkness and *phyE* mutants shared similar transcript changes that were distinct from light induction (Fig 2.3.4). The type A *ARRABIDOPSIS RESPONSE REGULATOR4* (*ARR4*) was greatly increased in transcript level between day 3 and 5 under light, while expression was only slightly increased in the *phyE* mutant or under

darkness. ARR4 has been shown to interact with PHYB under red light and cytokinin (CK) mediation (Mira-Rodado *et al*., 2007).

Cytokinin regulates auxin synthesis and vice versa (Jones *et al.*, 2010), and both hormones have been found to be involved in SE (reviewed by Gaj, 2004). Gaj also reported that several SE non-Arabidopsis SE systems require added CK, although in this system CK addition resulted only in callus formation with little to no SE. Auxin synthesis is also upregulated by *LEAFY COTYLEDON2* (*LEC2*) a SE promoting transcription factor (Stone *et al.*, 2001) expressed during light induced SE or with application of EBL (Supplemental Figure 2.A.1). *LEC2* expression is downstream of AGL15 signaling (Zheng *et al*., 2009).

As AGL15 activity promotes SE (Fig 2.3.5, Harding *et al*., 2003) it is likely highly transcribed during SE induction and only the wild type under light had enhanced *AGL15* at day 5 (Fig 2.3.4) compared to *phyE* or darkness. Partial restoration of *AGL15* transcripts in *phyE* and darkness occurred with EBL application. The differences in transcription of *ARR4, CPD, LEC2* and *AGL15* shared between darkness and phyE versus light during SE induction is evidence of SE specific transcriptional differences. Thus the highly increased transcription of *CPD* under light and no increase under darkness and in *phyE* strongly suggest BR involvement in SE induction. This supports the BR and BR-antagonist application results by Azpeitia *et al* (2003), Aydin *et al* (2006) and Belmonte *et al* (2010).

Unfortunately the BR receptor shown to associate with SERK1, BRI1 (Kalova *et al.*, 2006) does not seem to transduce the BR signal into the SE pathway as *bri1* is not significantly different from wild type in SE response (Fig 2.3.5). This does not exclude the role of BRs, but suggests the possibility of redundancy or independent BR signaling through other BR receptors. BRI1 activity is linked to SERK1-4 (Gou *et al.*, 2012), but the results suggest that in addition to mutation of the known SERK1 (Hetch *et al.*, 2001) the mutation of the SERK5, which has no known function, also reduces SE competence. Since BRI1 has not been shown to interact with SERK5, this suggests that other BR receptor(s) are likely responsible.

Mutation of SERK3 unexpectedly causes an enhancement to SE competence (Fig 2.3.5). Possible mechanisms that would cause this include: competitive reduction by SERK3 for dimerization with the BR receptor partner for SERK1/5, inactivation of SERK1/5 SE signaling by dimerization to SERK3, or independent SE inhibition by SERK3 signaling. Future studies would investigate whether SERK1/3/5 form dimers with each other. Identifying SERK5 dimer partners would come next as SERK5 has less demonstrated functions (Karlova *et al.*, 2006, Gou *et al.*, 2012) and thus potentially fewer partners unrelated to BR and SE.

While the Light -> BR -> SERK model failed to be proven, it has not been invalidated. Red light signaling through PHYE followed by up regulation of BR biosynthesis still holds true. Furthermore, BR application partially recovered *AGL15* expression. Bridging BR to AGL15 SE promoting activity, possibly through a BRL/SERK1/5 pathway, might contribute to our understanding of light induction during SE.

2.5 Materials and Methods

Plant Material and Growth Conditions

All *Arabidopsis thaliana* plants used were Columbia unless otherwise specified. Wild types were Col-0. SAIL (Sessions *et al.*, 2002), SALK (Alonso *et al.*, 2003) T-DNA insertion lines (Col-3 and Col-0 backgrounds respectively) and other mutant lines were obtained from the Arabidopsis Biological Resource Center. Homozygous progenitors were screened by PCR (Supplemental Table 4.A). Primers and insertion locations were obtained from Signal Salk (http://signal.salk.edu/). The phytochrome mutants were qualitatively verified for phenotypes previously demonstrated in literature (reviewed by Franklin and Quail, 2010); *phyA* mutants was selected for long hypocotyls under far-red light; *phyB* and *phyD* were selected for long hypocotyls under red light. The *bri1* mutant was verified to have a cabbage like vegetative growth and highly reduced fertility (Clouse *et al*., 1996). Seeds were surface sterilized with 75% ethanol and 0.1% Triton-X for 10 min, followed by a 95% ethanol wash for 1 min. Seeds were dried on sterile filter paper before being sown on solid media (½Murashige Skroog (MS) [plantMedia], 1% sucrose,

0.3% Phytagel [Sigma®]). To synchronize the seeds they were stratified at 4^{o}C for 3 days. Plates were germinated under 24 h light (warm white fluorescent [Philips] at 50±5 umol s^{-1} m^{-2}) and kept for two weeks at 22±2^{o}C. Seedlings were transferred into soil (Sunshine LA4 aggregate plus [Sungro®]) pots with individual plants separated by plastic dividers (Arasystem [Betatech]). Potted plants were grown in a growth chamber (16h/8h day night cycle, warm white fluorescent at 50±5 umol s^{-1} m^{-2}, 22^{o}/18^{o}C) and watered with tap water twice a week.

DNA Extraction

Explants were homogenized in salt extraction buffer (1.25 mM EDTA, 40 mM TRIS, 12.5 mM NaCl, 0.025% SDS) and then mixed with chloroform. The mix was separated by centrifugation (15k g, 5 min) and the top aqueous layer was transferred. DNA was precipitated out by adding one volume of isopropanol and centrifugation (15k g, 5 min). Supernatant was discarded and the pellet was air dried. Dry pellets were dissolved in diethylpyrocarbonate (DEPC) treated water and boiled to evaporate residual isopropanol and denature any DNAse contaminants (96^{o}C, 10 mins) before storage at -20^{o}C.

Somatic Embryogenesis Culture Conditions

Green siliques were surface sterilized in 75% ethanol for 5 min. Immature zygotic embryos were obtained by hand dissection of embryo sacs inside the siliques. Late stage embryos (large green cotyledons) were preferred as mortality was higher in younger explants. Explants were kept for 14 days on induction media (Supplemental 4.B) and then transferred to hormone-free, solid media (½MS, 1% sucrose, 0.3% Phytagel [Sigma®]) to mature for 7 days. When plant growth regulators (PGR) were applied to media, they were applied after auto-claving, except for 2,4-D and BA, which were added prior to auto-claving. PGRs were dissolved in 95% ethanol or filter sterilized water, depending on solubility. Controls for PGR treated media were treated with an equal volume of solvent. Unless otherwise stated induction was under 24 h light (warm white fluorescent at 50±5 umol s^{-1} m^{-2}) and maturation was under 24 h darkness both at 22±2^{o}C. Somatic embryos were scored based on the following criteria: Identifiable, distinct apical and basal regions; independence of epidermis and vascular system from the original explant or other new

formed somatic structures. Scoring was done by physically separating every potential embryo by hand and inspection under microscope. Each experiment was trialed once.

Somatic Embryogenesis Light Treatments

Somatic embryogenesis induction plates were subject to light treatments (color: 30±2, white: 50±5 umol s^{-1} m^{-2}) as specified inside a dark room (22±3^{o}C). The lights used were as follows: 660 nm Deep Red [LedEngin LZ4], 740 nm Far Red [LedEngin LZ4], 540 nm Green [Led Engin LZ4], 450 nm Royal Blue [Cree XTE], Warm white [Philips F32TB/TL841]. Darkness was simulated by wrapping plates in aluminum foil.

RNA Extraction

RNA extractions were modified from the LiCl method described by Onate-Sanchex and Vicente Carbajosa (2008). Polyvinylpyrrolidone-40 (4% $_{w/v}$) was added to the extraction buffer (0.4 M LiCl, 0.2M TRIS, 25mM EDTA, 1% SDS) to protect against phenolics and reduce phenol contamination. Homogenized samples were phase separated by 1:1 volume chloroform and centrifugation (15k g, 5 min). The aqueous layer was re-separated by 1:1 volume of phenol and 2/5 volume of chloroform and centrifugation (15k g, 5 min). RNA was precipitated overnight (-20^{o}C) by increasing LiCl concentration to 2 M. After thawing the solution the RNA was pelleted by centrifugation (15k g, 30 min). After discarding the supernatant the pellet was then dissolved in 500 µl DEPC treated water. Carbohydrates were removed by precipitation (7 µl Na acetate, 250 µl EtOH) with centrifugation (15k g, 10 min). Supernatant was transferred and RNA was ethanol precipitated (50 µl Na acetate, 500 µl EtOH) at -20^{o}C for one hour. RNA was pelleted by centrifugation (15k g, 30 min). After discarding the supernatant the pellet was dissolved in 20 µl DEPC treated water. RNA extraction for non-seed explants skipped the first chloroform separation step. RNA concentration and quality was determined by nanodrop [Biorad®] after dissolving of recovered RNA in DEPC treated water. Good quality was a value $\geq$ 2 for the 260/230 ratio and $\geq$ 1.8 for the 260/280 ratio. DNA was removed from samples using 10 units of DNase I recombinant (Roche®) at 37^{o}C for 1 hour. Dnase was inactivated by boiling (96^{o}C, 15 min) RNA samples were stored at -80^{o}C.

PCR and RT-qPCR

PCR was carried out using a (1:2:6:6) mixture of DNA template, primer, water and BioMix [Bioline®]. Confirmation reactions for SALK lines were run using the following cycle; 94^0C (5 min) {94^oC (30 s): 60^oC (30s): 72^oC (45s)} x36 cycles. SAIL & GABI-KAT lines were run with a 56^oC annealing temperature.

Reverse transcriptase reactions were performed following the Reverse Transcription System (Promega®, A3500) protocol for 2 µg of RNA using random hexamers (1 h at 37^oC followed by 5 min at 85^oC). RT-qPCR was done on a CFX96 (Biorad) with Sso-fast™ Evagreen® (Biorad) using the following cycle; 95^oC (2 min), {95^oC (3s): 60^oC (7s)} x39 cycles. Following the protocol from Biorad each reaction was an 8 µl mix consisting of 4 µl of Evagreen, 1 µl of primer mix and 3 µl of cDNA. All primers were designed to have a Tm at 60^oC (Supplemental Table 4.A.2).

Real Time Analysis

All samples were pooled from 150 (day 3 SE induction) or 50 (day 5 SE induction) individuals. Each experiment had three biological replicates. Cycle threshold (Ct) values were taken as the average of three technical replicates and outliers were thrown. All Ct values were normalized to a housekeeping gene (*UBQ10*) using the ΔΔCT method and corrected for primer efficiency (Livak and Schmittgen 2001). Primer efficiencies were calculated from the slope of four 4x serial dilutions for each gene.

Statistical analysis

All statistical analysis was performed with one-way ANOVA tests. Somatic embryogenesis data was tested after normalization to the control. Real-time PCR data was tested after ΔΔCT (Livak and Schmittgen 2001) normalization and before logarithmic transformation. SigmaPlot© [Systat®] was used for calculations.

CHAPTER 3: THE WOX7 DARK GERMINATION PHENOTYPE

3.1 Abstract

Dark germination is a poorly studied field in developmental biology. Dark germination in Arabidopsis (Col-0) was found to require nitrate signaling. Nitrate induced germination was discovered to be enhanced by cytokinin while examining the *wox7* mutant. The *wox7* mutant does not germinate in the dark, but germinates 100% under continuous light. The *wox7* phenotype was rescued by high cytokinin co-application with high nitrate. *WOX7* expression was not detected during dark germination, but was present during seed development. The endosperm was required to suppress germination in both wild type and *wox7* implicating *PHYB* germination control. The results suggest that nitrate and cytokinin induce germination through PHYB activity and *WOX7* may indirectly modulate nitrate based low cytokinin response or Pfr PHYB activity in the seed.

3.2 Introduction

Seed germination is a vital process for both plants and humans. Human civilization based on agricultural production has relied on the storage and sowing of seed crops for millennia (reviewed by Meyer *et al*., 2012). For sessile plants, seeds serve as a means for progeny to find new opportunities and avoid death from growing in unfavorable situations. To avoid such pitfalls, the seed has evolved to not only provide protection, but has adapted to sense suitable environments. Waiting for favorable conditions before germinating is one commonly conserved strategy (reviewed by Finch-Savage and Leubner-Metzger, 2006).

The minimal requirements for germination in *Arabidopsis thaliana* are light, oxygen and water (reviewed by Rajjou *et al.*, 2012). Just oxygen and water has been demonstrated to be sufficient for germination in the Landsberg *erecta* (Shinomura *et al*., 1994), when after-ripened for 2 months after harvest and cold stratified (prolonged exposure to low temperatures). Even with after-ripening and/or cold stratification, oxygen and water were insufficient to induce dark germination in the Columbia ecotype (Papi *et al*., 2000, Bethke *et al*., 2007).

Seed embryos of Col-0 were demonstrated to germinate under darkness on water agar when freed from the aleurone (endosperm) of the seed coat (Bethke *et al*., 2007). The aleurone imposes dormancy, as dissected embryos from dormant varieties of Arabidopsis readily germinate. Aleurone suppression was also partially reduced by nitric oxide (NO) application (Lee *et al.*, 2010). They further discovered that a diffusible factor from the coats of dormant seed suppressed growth of coat-free embryos. Their work revealed that only coats from dormant seeds produced abscisic acid (ABA) and ABA alone was sufficient to suppress embryo growth.

Phytohormone antagonism between ABA acting as a suppressor and gibberellic acid (GA) as a promoter of germination serves as the classical backbone for subsequent extensions of the germination-dormancy model (reviewed by Finch-Savage and Leubner-Metzger, 2006). As many reviews covering the latest molecular model are available in literature; only major key points will be presented. Residual and *de novo* ABA synthesis in dormant seed maintain the activity of ABA response factors identified as the ABSCISIC ACID INSENSITIVE (ABI) gene set. ABI activity restricts germination, in part by suppressing GA levels. GA degrades DELLA factors that up regulate ABI transcription. In addition to removing blocking of germination by ABA, GA also activates germination events such as cell wall loosening. GA biosynthesis is up-regulated by light, a basic germination promoting signal (reviewed by Rajjou *et al.*, 2012).

Light is one a major abiotic determinant of germination. Red light reception by PHY leads to repression of PHYTOCHROME-INTERACTING-FACTOR1 (PIF1/PIL5) (Oh *et al.*, 2007). Their work also demonstrated that PIFs maintained DELLA transcription. PIF1 also reduces GA levels and increases ABA accumulation (Oh *et al.*, 2005). Seed ABA homeostasis control is mediated by PHYs irrespective of light (Shinomura *et al.*, 1994; Lee *et al.*, 2012).

PHYA to E (reviewed by Bae and Choi, 2008) are the principle red/far-red photoreceptors in *Arabidopsis* (except WS-2, which lacks D). Photo-isomerization between the red light absorbing Pr and the far-red light Pfr conformations changes the protein-protein interactions of phytochrome. Pfr PHY have been shown to regulate translation in the cytosol through phosphorylation of PENTA1 (Paik *et al*., 2011) and causing transcriptome changes in the nucleus by interaction with PIFs. PIF3 binding leads to the nuclear translocation of Pfr PHYB (Pfeiffer *et al.*, 2012).

In the Landsberg (Ler) *PHYB* is the principle phytochrome involved in short pulse red light and dark germination (Shinomura *et al.*, 1994). Red, far-red short pulse and dark germination was highly reduced in the *phyB* loss of function mutant. Overexpression of *PHYB* in the Nossen increased far-red and dark germination to red light levels (~100%) (McCormac *et al*., 1993). Continuous light germination is mediated by *PHYE* and *PHYA* (Hennig *et al.*, 2002). PHYA activity is required for coatless seed embryos to resume growth (Lee *et al.*, 2012). Their work also demonstrated that seed coat control of germination was found to be due to PHYB activity. Red light shift of PHYB to the Pfr conformation was shown to be stabilized by ARABIDOPSIS RESPONSE REGULATOR 4 (ARR4) (Sweere *et al.*, 2001).

ARRs are terminal phosphorylation targets of the cytokinin (CK) two-component phosphor-relay system. CKs are a family of phytohormones involved in meristem activity, reproduction, senescence as well as light and nutrient response (Reviewed by Brenner *et al.*, 2012). Responses are elicited through phosphorylation changes of ARRs of which three categories have been defined; Type A – negative feedback response regulators, that lack transcriptional activity; Type B – positive feedback response regulators that bind to DNA with transcriptional activity; and CYTOKININ RESPONSE FACTORs (CRFs) – redundant transcriptional response factors (Reviewed by Brenner *et al.*, 2012).

The role of CKs in germination is complex. While ectopic CK does not promote germination it has been shown to antagonize exogenous ABA based repression (Wang *et al.*, 2011). Down regulation of ABA response factors and deactivation of ABI5 by type-A ARR (4, 5 and 6) interaction was determined as a putative cause. CK has also been shown to rapidly induce NO production in Arabidopsis cell culture (Tun *et al*., 2001). NO is a

molecule that stimulates germination and reduces dormancy (Bethke *et al.*, 2005), However exogenous CK also delays light induction of germination, as faster germination was observed in higher order mutants of the CK receptors (AHK/CRE1) when grown on sugar-free Murashige and Skoog (MS) media (Riefler *et al.*, 2006). The same effect was seen during dark, red or far-red light germination. MS contains high level of nitrogen, and CK biosynthesis has been shown to be up-regulated by nitrate (Takei *et al.*, 2004, Garnica *et al*., 2010).

Nitrate is a major source of inorganic nitrogen utilized by plants to form organic nitrogen compounds via the nitrate reductase (NR) pathway. In addition to nutrient responses, nitrate also initiates signaling. Addition of nitrate was observed to increase CK levels in Arabidopsis roots (Takei *et al.*, 2004) as well as in wheat shoots (Garnica *et al*., 2010). Cytokinin signaling was found to relay from the roots to the shoot and back to the root to control lateral root development in response to nitrate availability (Ruffel *et al*., 2011). In their review, Sakakibara *et al* (2006) note that nitrate signals use both cytokinin dependent and independent signaling, although the nitrate sensor or signaling components are unknown.

In their study, Alboresi *et al* (2005) found nitrate was able to stimulate germination, whether it was applied or residual in the seed, whereas glutamine and potassium did not. Additionally they found that the dual affinity nitrate transporter (NRT 1.1) was required for low nitrate germination, but as high dosage rescued the mutant phenotype there is likely to be separate high nitrate signaling.

Seeds of *wox7* mutants in *Arabidopsis thaliana,* Columbia (Col) had reduced germination on callus induction media under darkness. *WOX7* is an uncharacterized member of the 15 *WOX* transcription factors, and part of the *WUSCHEL* (*WUS*) clade (reviewed by van der Graff *et al.*, 2009). WUS was first discovered as a required component of meristem maintenance in the shoot apex. Other characterized members have since been found to be involved in meristem and organ formation as well as transitioning and patterning all over the plant body. There is no known relationship between germination and *WOX* genes.

3.3 Results

Nitrate Signalling is the Minimal Requirement for Dark Germination in Col

Under continuous light on ½ MS + 1% sucrose, *wox7* germinates 100%. On modified MS callus induction media, *wox7* mutants was discovered to have reduced dark germination (qualitative assessment). As the initial discovery of the germination difference was on callus induction media, it was necessary to isolate the stimulatory factor(s).

Callus induction media contained 3 μM of the synthetic CK Benzoic Acid (BA), 3 μM of the synthetic auxin 2,4-D, 25 mM sucrose and ½ MS nutrients. The major components of ½ MS are nitrate (20 mM), ammonium (10 mM) and potassium (10 mM). Dark germination for Col-0 was near 0% on water agar plates and not significantly increased by any major media component but nitrate (Fig 3.3.1). Nitrate at 20 mM produced a dark germination level of about 55% after 4 days after stratification and was about half of that by the 2nd day.

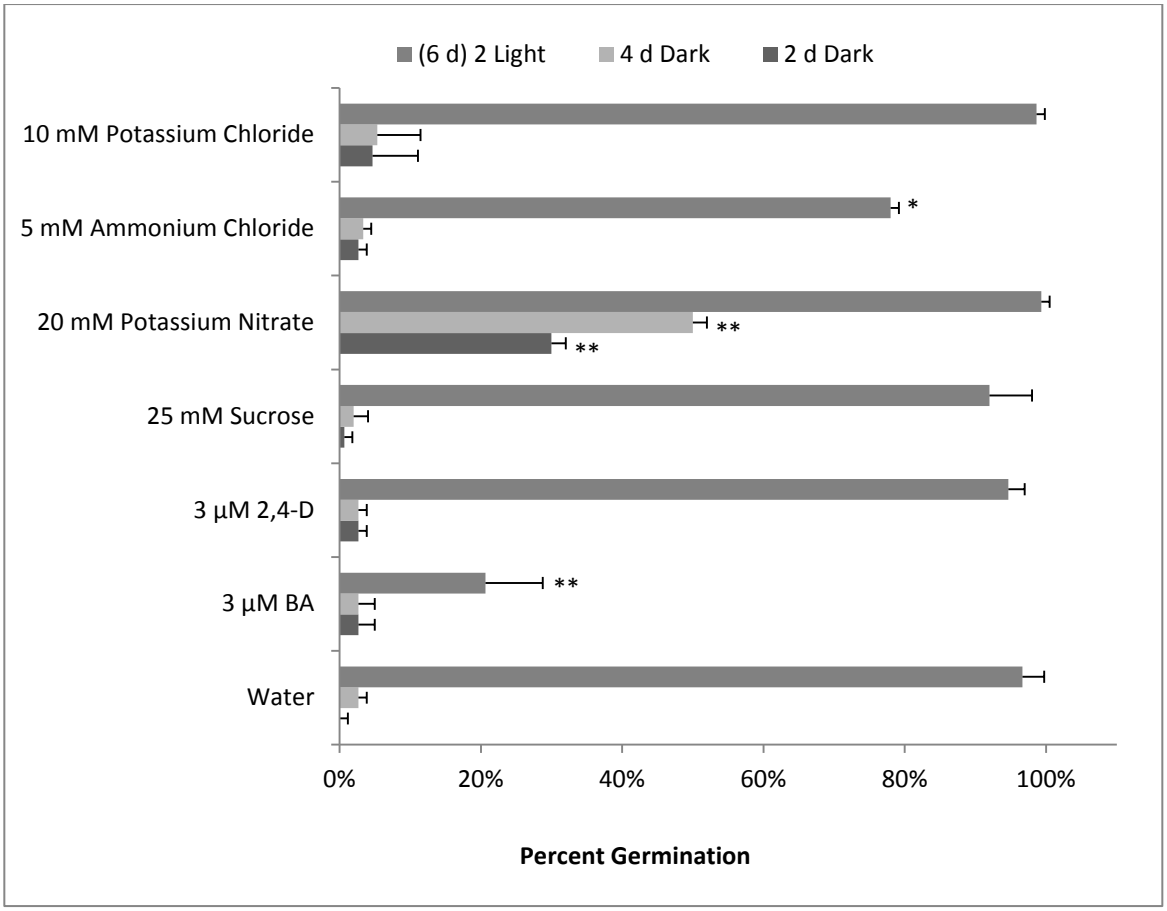

Fig 3.3.1 Dark Germination Response of Arabidopsis to Media Components
Three replicates of 50, 2 month old seeds of Col-0 were stratified (4°C, 3 d) in darkness and scored for germination ± standard deviation after 2 and 4 days in darkness and 2 more days in continuous light at 22°C. Seeds were placed on 0.8% agar containing a single major component of callus induction media; KCl, NH_4Cl, KNO_3, sucrose, 2, 4-D, BA or water. The NH_4Cl treatment was lowered to 5 mM and pH 6.8 due to potential toxicity. Significance of difference from water, *: $p \leq 0.05$, **: $p \leq 0.001$ (One-way ANOVA)

Arabidopsis thaliana Columbia Dark Germination Response to Nitrate Concentration Exhibits a High and Low peak

As nitrate requirements for dark germination in Col-0 was not available from literature a range of NO_3 concentrations were assayed. Increasing nitrate seemed to correlate with decreasing germination rate at day 2 at concentrations higher than 1 mM of nitrate. Nitrate concentrations had relatively decreased dark germination at day 4 outside the two peaks at 55% and 65% dark germination observed at 5 mM and 20 mM of KNO_3; although the true peaks might have not been included in measurements.

The exact concentration to response is expected to vary between seed lots as Alboresi *et al* (2005) had previously demonstrated that endogenous seed nitrate was dependent on the mother plant; variation in seed nitrate changed germination response to exogenous nitrate. Therefore all following tests on Col-0 reported are from the seed lot used in this experiment.

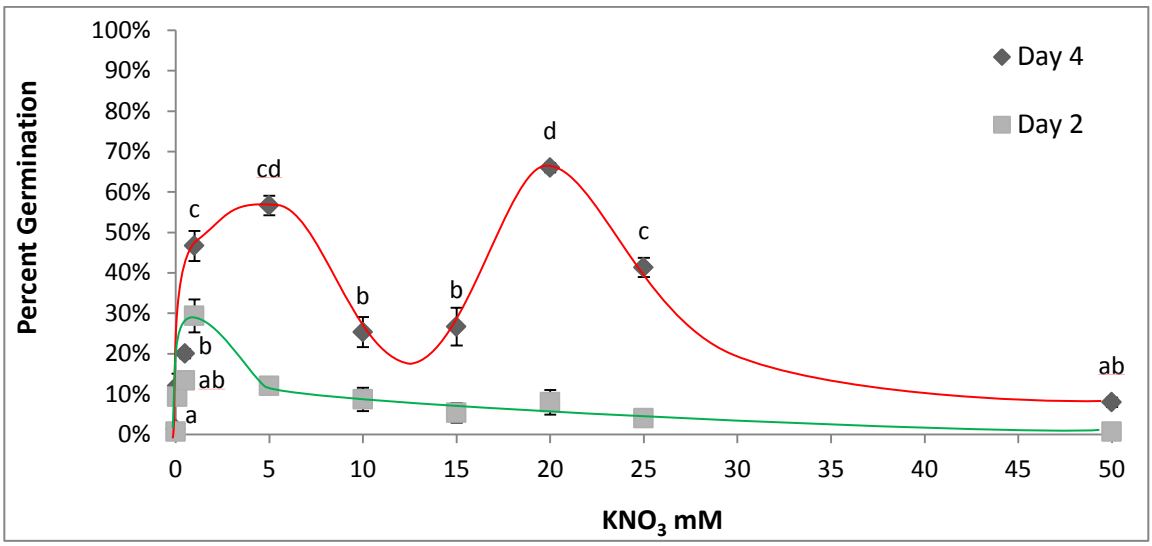

Fig 3.3.2 Dark Germination Response to Nitrate Concentration

Three replicates of 50 seeds of 6 month old Col-0 were stratified (4°C, 3 d) in darkness and scored for germination ± standard deviation after 2 and 4 days in darkness. Seeds were placed on 0.8% agar containing increasing nitrate concentration: 0, 0.1, 0.5, 1, 5, 10, 15, 20, 25, 50 mM. Best fit lines drawn by hand for illustrative purposes only (cannot be used for value interpolation). Statistically distinct groups ($p \leq 0.05$) are denoted a, b, c and d for day 4. (One-way ANOVA)

The wox7 Mutant Germinates Normally under Continuous Light, but Does Not Germinate under Darkness and is Partially Rescued by Co-Application of Cytokinin with Nitrate

After finding that nitrate was sufficient to induce dark germination, the *wox7* phenotype was confirmed using both nitrate peaks (Fig 3.3.2) at 5 and 20 mM for T4 SALK (Alonso *et al.*, 2003) *wox7* seeds. Surprisingly nearly no germination was observed compared to just being visibly reduced in callus media (qualitative observation). The other components of the media were tested in co-application; sucrose or cytokinin as a promoter (Wang *et al.*, 2011). These co-application trials were performed on T5 SALK seeds using both the SALK and an independent SAIL (Sessions *et al.*, 2002) *wox7* mutant (Fig 3.3.3).

Nearly 0% germination was observed for the *wox7* mutants on 20 mM nitrate, whereas the WT was around 50% (Fig 3.3.3). The replicated phenotype in the independent SAIL mutant suggests that secondary insertion(s) effects are unlikely. Cytokinin rescued the *wox7* phenotype and enhanced nitrate mediated dark germination in the wild type for both the rate (day 2) and final levels (day 4). Light germination (Fig 3.3.1) was however antagonized by CK without nitrate. Cytokinin co-application rescued *wox7* to wild type levels, but CK rescue of *wox7* was highly suppressed by sucrose, which did not significantly reduce CK enhancement of WT germination. All plates reached approximately 100% germination when placed under continuous light for 2 days.

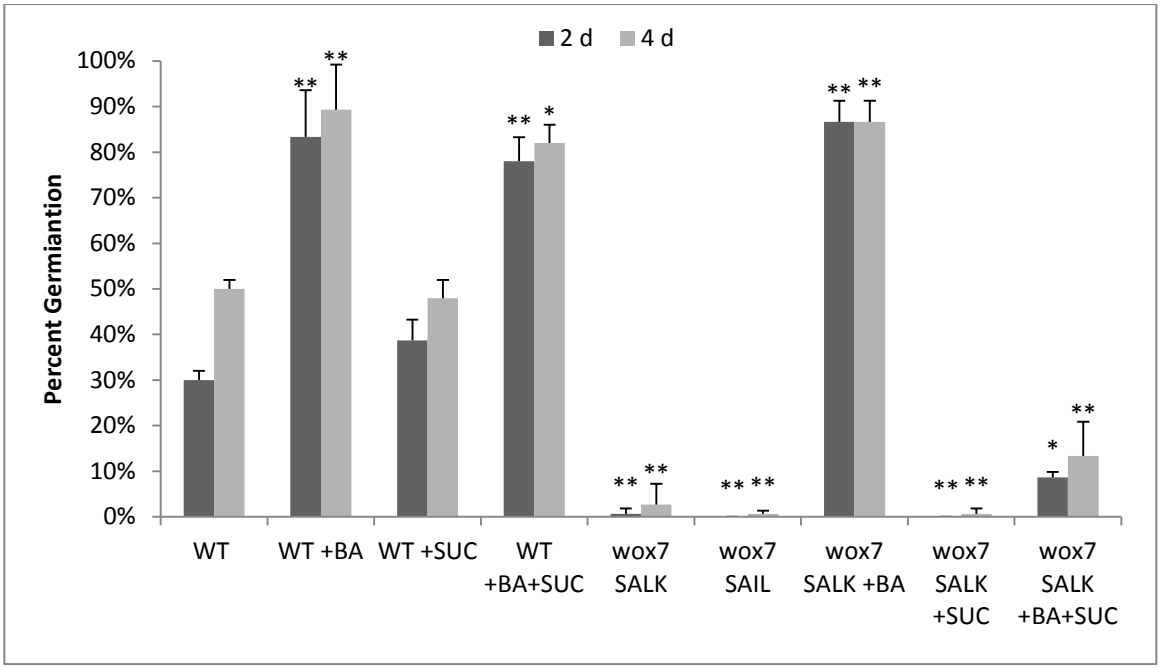

Fig 3.3.3 Dark Germination Response of wox7

Three replicates of 50, 2 month old seeds of Col were stratified (4°C, 3 d) in darkness and scored for germination ± standard deviation (SD) after 2 and 4 days in darkness. Wild type Col-0 (left) used as a control for the dark germination phenotype of *wox7* mutants (right). An independent *wox7* mutant from the SAIL line (Col-3) was also assayed. All plates contained 0.8% agar and 20 mM KNO_3 in addition to the indicated treatment(s): BA- 3 μM BA, SUC- 25mM sucrose. Significance of difference of versus WT, *: $p \leq 0.05$, **: $p \leq 0.001$ (One-way ANOVA)

Cytokinin Enhancement of Nitrate Induced Dark Germination is Concentration Dependent

Cytokinin was more interesting than sucrose, as the former could rescue the *wox7* phenotype. The effect of CK concentration was determined and both the high/low nitrate peaks were examined (Fig 3.3.4).

Interestingly, the dark germination response to CK application could be suppression or enhancement depending on the concentration (Fig 3.3.4). The CK level in the callus media had fortunately been in the enhancement range (3 μM), as 1 μM and 5 μM BA resulted in reduction of dark germination. While dark germination was not obviously different between the high/low nitrate only applications, in both cases around 60%, the differential response to CK was obvious. CK enhancement was strong at 20 mM NO_3, whereas CK co-application did not have any real positive effect with 5 mM NO_3.

At 3 μM BA and 20 mM KNO_3, the 6 month (Fig 3.3.4) old seeds germinated slower than 2 month old seeds (Fig 3.3.3), around 5% versus 80% at day 2, but reached similar levels at day 4, around 90% dark germination. As the maximal germination induction by CK plus nitrate was similar between 2 and 6 months of dry storage for the wild type dormancy is likely completely overcome by CK plus nitrate. Oddly the day 4 dark germination of *wox7* seeds had diminished from about 90% at 2 months (Fig 3.3.3) to 60% at 6 months (Fig 3.3.5). This did not seem to be due to a loss of seed vigor as both wild type and wox7 seeds reach 100% germination under continuous light (qualitative observation).

The *wox7* mutants did not respond below or above the high CK peak (Fig 3.3.5). This would suggest that *wox7* does not simply shift CK sensitivity, but rather is insensitive to the (putative) low CK pathway altogether. The lack of response at 1 μM and 5 μM BA suggests that the high CK response at 3 μM is a very specific phenomenon, possibly an *in vitro* artifact resulting from competing, dose dependent CK responses.

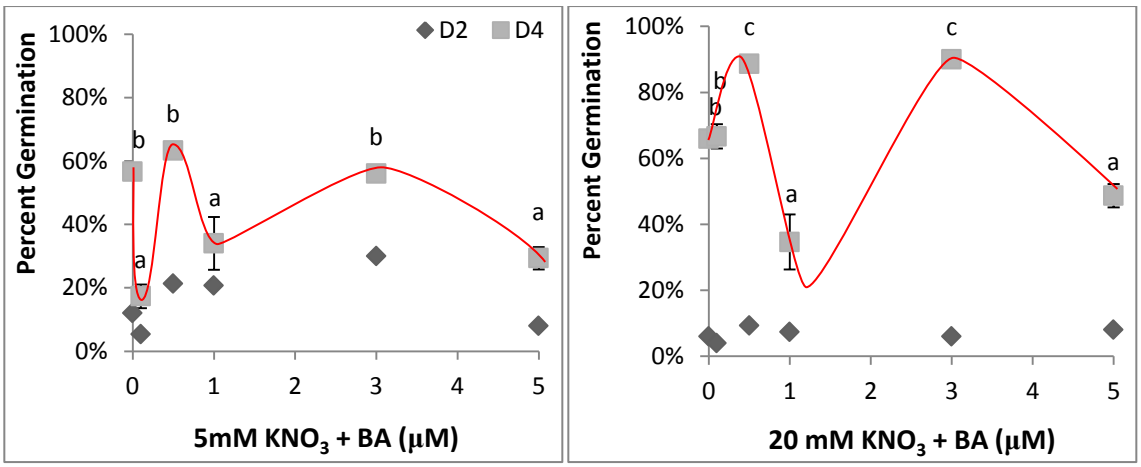

Fig 3.3.4 Dark Germination Response to Cytokinin Concentration
Three replicates of 50, 6 month old seeds of Col-0 were stratified (4°C, 3 d) in darkness and scored for germination ± standard deviation after 2 and 4 days in darkness. The low 5 mM (left) and high 20 mM (right) KNO_3, 0.8% agar plates were supplemented with different concentrations of BA: 0, 0.1, 0.5, 1, 3, and 5 µM. Statistically distinct groups ($p \leq 0.05$) are denoted a-c for day 4. (One-way ANOVA, pair-wise)

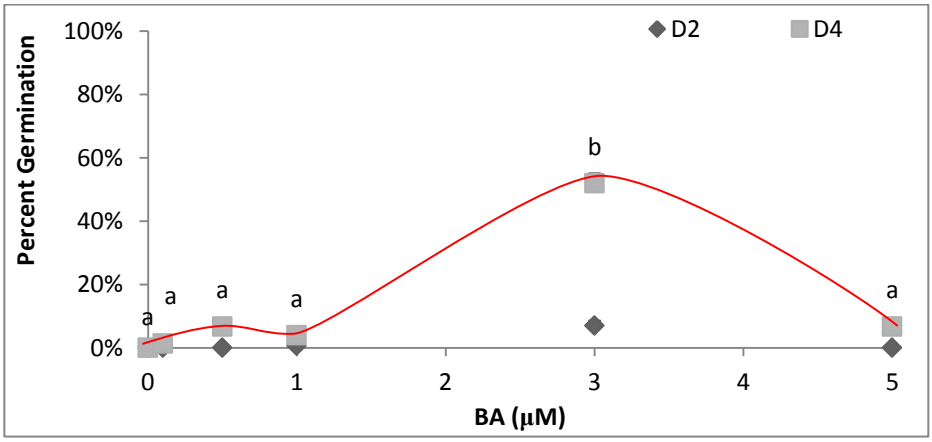

Fig 3.3.5 Dark Germination Response of wox7 to Cytokinin Concentration
Three replicates of 50, 6 month old seeds of SALK *wox7* were stratified (4°C, 3 d) in darkness and scored for germination ± standard deviation after 2 and 4 days in darkness. 20 mM KNO_3, 0.8% agar plates were supplemented with different concentrations of BA: 0, 0.1, 0.5, 1, 3, and 5 µM. Statistically distinct groups ($p \leq 0.05$) are denoted a-b for day 4. (One-way ANOVA, pair-wise)

WOX7 Expression is Not Detected During the Process of Germination, but is Present in the Embryo/Testa and the SAM During Cytokinin/Nitrate Induced Cotyledon Opening

As the next step, GUS localization was done using the SAIL *wox7* line. A homozygous SAIL T5 line was isolated by progeny testing of single-seed descent T6 plants for pWOX7::GUS expression in the silique. GUS staining of the siliques showed expression in germinated pollen (A), chalazal end of the pre-globular stage testa (B) and the endosperm region of the testa at the heart/torpedo stage (C) (Fig 3.3.6). Dissected embryos did not show GUS activity.

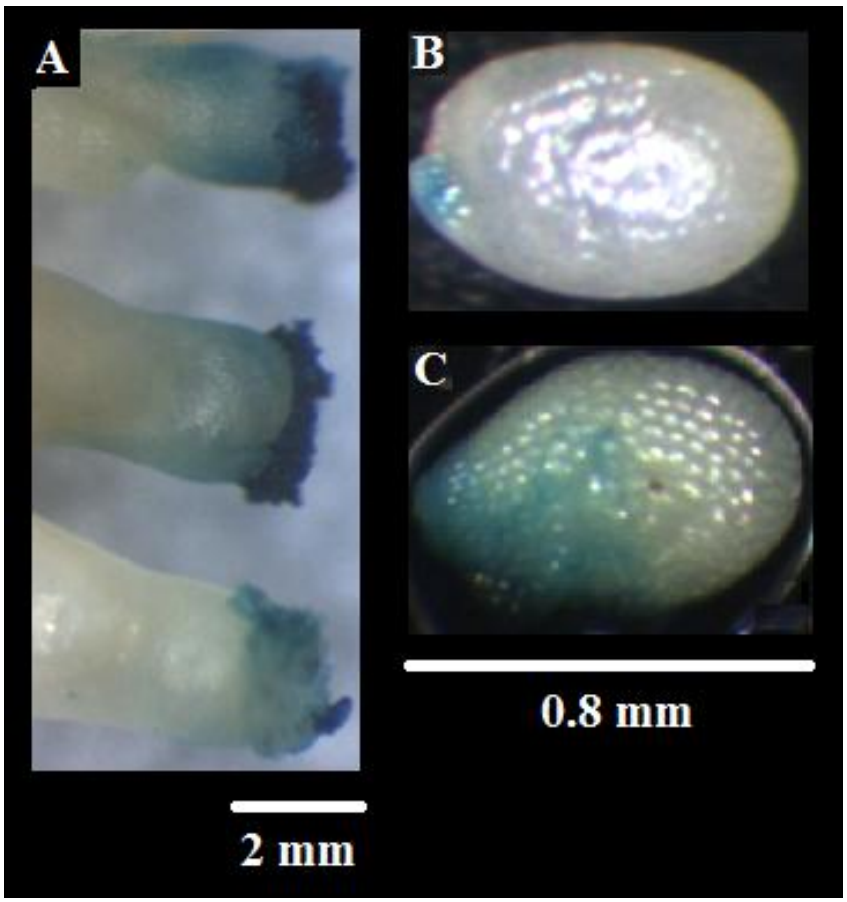

Fig 3.3.6 WOX7 Expression During Seed Development
pWOX7::GUS expression of during seed development. A) Germinated pollen expression of *WOX7* at the pre-globular (top), heart/torpedo (middle) and late stage of embryo development (bottom). B) Pre-globular embryo sac expressing *WOX7* at the chalazal region of the testa. C) Embryo sac at the heart/torpedo stage expressing *WOX7* in the endosperm region of the testa. Consistent GUS expression is lost by the bent cotyledon stage.

Surprisingly there was no detectable expression anywhere during germination (Fig 3.3.7) even when seeds were germinated without a coat, where 100% germination occurs even without treatment in both wild type and *wox7* at day 2 (qualitative). Embryos germinated on high nitrate (20 mM) had opened cotyledons and pWOX7::GUS activity in the shoot apex, whereas cotyledons were less open on water agar (Supplemental Figure 3.B.1). Between the open cotyledons and on the SAM there was strong pWOX7::GUS expression. The *wox7* mutant did not affect cotyledon opening response to high nitrate.

It was possible that GUS was insufficiently sensitive (with 24 hours incubation), so RT-PCR was used for confirmation. No *WOX7* mRNA was detected for 10 ng/µl RNA samples at 0, 1 and 2 days post stratification (Supplemental Figure 3.A.1)**.** Over 60% of the seeds were germinated at 4 days, which made RT-PCR unreliable as germinated seeds expressed pWOX7::GUS in the SAM (Supplemental Figure 3.B.1) and was omitted. The lack of *WOX7* expression during germination (Fig 3.3.7) did not support the hypothesis of a simple germination component malfunction. Fortunately *WOX7* was expressed during seed development (Fig 3.3.6), so a more complex hypothesis where *WOX7* affected seed development causing the *wox7* phenotype was adopted. Only one known mutant, *phyB,* has been reported with the same phenotype as *wox7*; this mutant had 100% germination under continuous light, nearly 0% under darkness (Shinomura *et al.*, 1994).

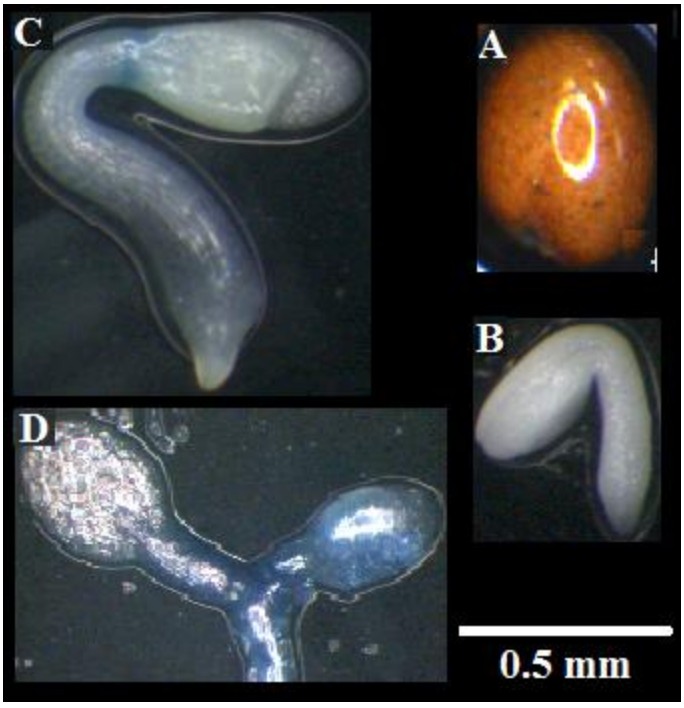

Fig 3.3.7 WOX7 Expression During Dark Germination

pWOX7::GUS expression of the SAIL *wox7* mutant during dark germination on 20 mM KNO_3 agar plates. A) seed coat and B) embryo prior to radicle extension. C) 48h after dissection from seed coat during radicle extension with 20 mM KNO_3. D) 96 h after dissection from seed coat during dark cotyledon opening of dissected embryos induced by 20 mM KNO_3.

The wox7 Germination Response to Short Light Pulses Resembles phyB

As the original *phyB* studies were in Landsberg erecta (Ler), and the mutant used was a homozygous SALK *phyB* in the Col-0 background it was necessary to determine if the loss of function germination phenotypes were conserved. As previously reported by Shinomura *et al* (1994) *phyB* seeds almost did not germinate with 20 mM nitrate in the dark or with short red light pulses (Fig 3.3.8). All *phyB* treatments reached ~100% germination after 2 additional days under continuous light (qualitative), which Shinomura *et al* (1994) had also found.

The loss of germination response to 20 mM nitrate dark induction and 15 min of red or green light pulses, which induced around 60%, 90% and 90% germination in the wild type, was observed in both *wox7* and *phyB* mutants. Under a longer 1 hour red or green light pulse, unlike *phyB*, *wox7* seeds had some germination response, around 50% to 60% compared to about 100% in the wild type. The germination speed comparing day 2 to day 4, was also reduced in *wox7*, which had only reached 40% of the day 4 germination level at day 2 compared to about 100% in the wild type.

The wox7 mutant also did not respond to 15 min short pulse far-red or blue light. However these treatments reduced nitrate induced dark germination in the wild type from 60% to around 40%. Since *wox7* and *phyB* had nearly 0% response to only nitrate, further examination of far-red or blue germination responses was not undertaken.

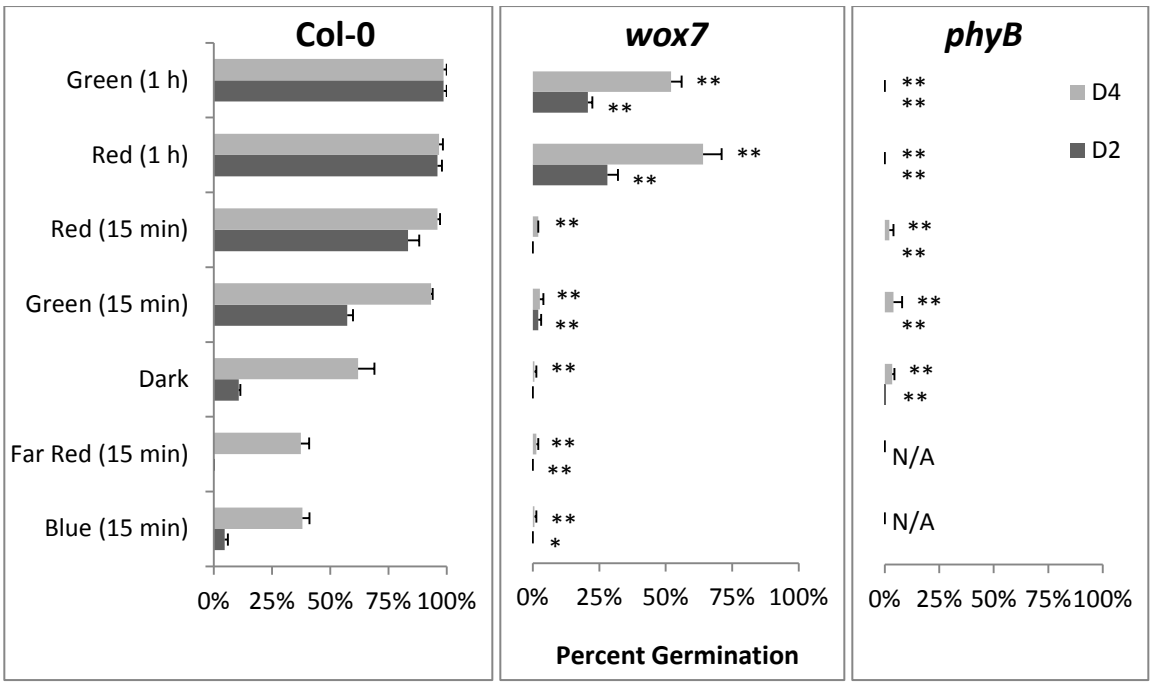

Fig 3.3.8 Dark Germination Response of Col-0, wox7 and phyB to Short Light Pulses

Three replicates of 50 of Col-0 (left), *wox7* (middle) and *phyB* (right) seeds were stratified (4°C, 3 d) in darkness and scored for germination ± standard deviation after 2 and 4 days in darkness (22°C). All plates contained 20 mM KNO_3 and 0.8% agar. Each plate was irradiated with indicated light (30 umol s^{-1} m^{-2}) for 15 mins or 1 hour post stratification: Red- 660 nm, Far Red- 740 nm, Green- 540 nm, Blue- 470 nm. *phyB* was not tested (not applicable, N/A) for Far Red or Blue. Significance of difference from Col-0 of same treatment *: $p \leq 0.05$, **: $p \leq 0.001$ (One-way ANOVA, pair-wise)

Since *phyB* like *wox7* did not dark germinate in response to nitrate alone, it was necessary to see if *phyB* could be rescued by CK co-application like *wox7* (Fig 3.3.3). The *phyB* mutant seeds did not respond to CK co-application with nitrate (Fig 3.3.9). This suggests that PHYB likely acts downstream of both nitrate and cytokinin.

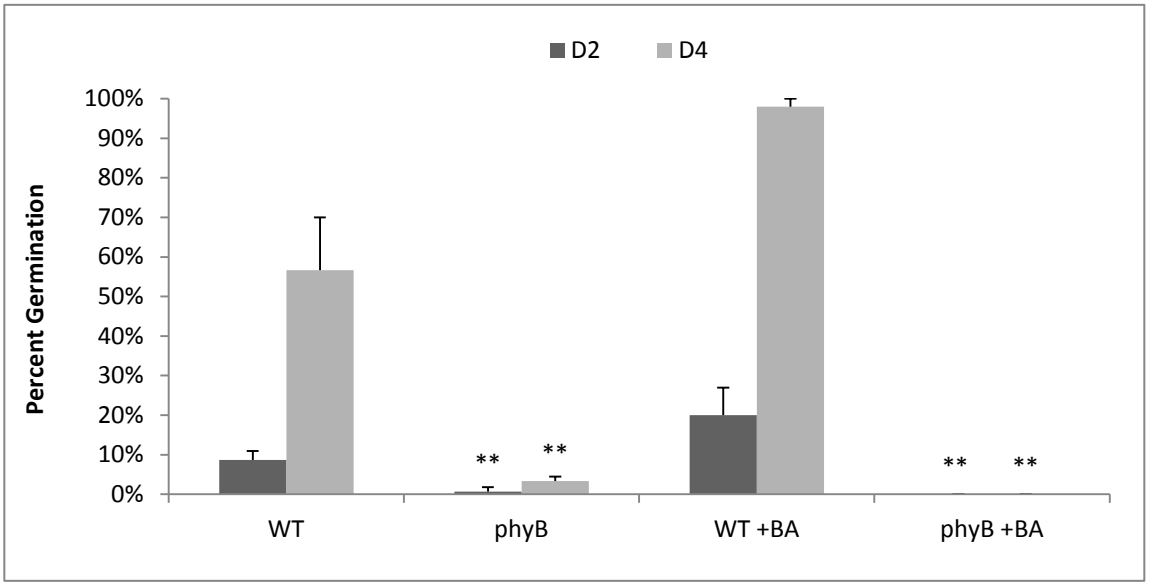

Fig 3.3.9 Dark Germination Response of phyB to Cytokinin and Nitrate
Three replicates of 50 seeds of Col-0 (WT) and *phyB* (Col-0) were stratified (4°C, 3 d) in darkness and scored for germination ± standard deviation after 2 and 4 days in darkness (22°C). All plates contained 20 mM KNO_3 and 0.8% agar in addition to the indicated treatment(s): +BA - 0.5 μM BA. Significance of difference from WT, *: $p \leq 0.05$, **: $p \leq 0.001$ (One-way ANOVA)

Nitrate Signalling is Independent of Nitrate Reductase

Since nitrate reductase (NR) activity directly metabolizes nitrate to nitrite and may be involved in germination production through putative NO biosynthesis, the genetic relation between nitrate induced dark germination and NR was examined (reviewed by Rajjou *et al.*, 2012).

The dark germination of the NR null activity Ler mutant *nia1/nia2* (Wang R *et al.*, 2004) was stimulated by high nitrate (Fig 3.3.10). 3 μM of CK co-applied with 20 mM nitrate increased *nia1*/*nia2* dark germination levels at day 4 from around 75% in the wild type to 100% in the mutant. Germination rate was also increased; the day 2 level was almost 100% of the day 4 level compared to about 50% in the wild type.

With 0.5 μM of CK germination was slightly reduced in *nia1*/*nia2* compared to wild type (Fig 3.3.10) from 75% to 60% after 4 days. Germination speed was greatly reduced from a day 2 versus day 4 level of 70% in the wild type to 3% in the mutant.

New Ler wild type seeds were not available as the plants had been killed by a problem in the green house, so an old seed lot (1.5 years) had to be used. Ler seeds have low dormancy and can dark germinate on water agar unlike Col (Fig 3.3.10). Dark germination was enhanced by nitrate, but not co-application of CK. This may possibly be due to the effects of prolonged after-ripening.

Since PHYB is upstream of GA the inability of nitrate and co-application of CK to overcome the non-germination of Ler *ga1-3* mutant (Koornneef and van der Veen 1980) was confirmed as expected (Fig 3.3.10). Indeed GA application at 1 μM fully rescues the dark germination phenotype of *wox7* and *phyB* (Qualitative).

Due to storage duration differences, the wild type could not be directly compared to the mutants. Fortunately the dark germination responses of the mutants alone was sufficient, as the results simply indicate that NR activity is not required for nitrate induction or cytokinin enhancement and GA biosynthesis is downstream of both.

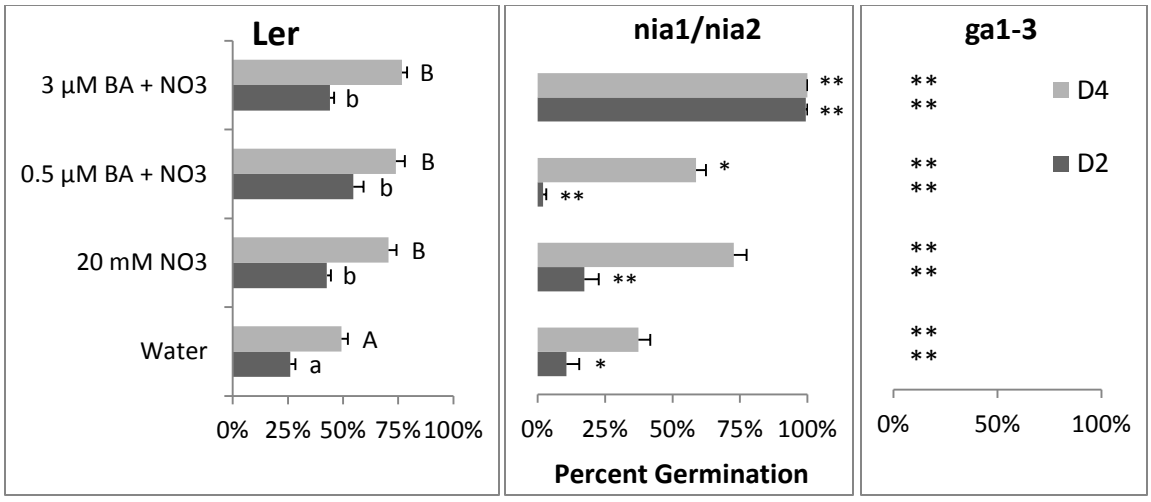

Fig 3.3.10 Dark Germination Response of nia1/nia2 and ga1-3

Three replicates of 50, 1.5 year old Ler (left), 2 month old *nitrate reductase* mutants (*nia1/nia2,* middle) and 2 month old *ga requiring 1* (*ga1-3*, right) seeds were stratified (4^{o}C, 3 d) in darkness and scored for germination ± standard deviation after 2 and 4 days in darkness (22^{o}C). Plates contained 0.8% agar and : 3 µM BA + 20 mM NO_3; 0.5 µM BA + 20 mM NO_3; 20 mM NO_3; water. Statistically distinct groups for Ler WT (left) ($p \leq 0.05$) are denoted a-b for day 2 and A-B for day 4. Significance of difference of *nia1/nia1* (center) and *ga1-3* (right) from Col-0, *: $p \leq 0.05$, **: $p \leq 0.001$

3.4 Discussion

Due to the lack of literature the basic dark germination requirements of *Arabidopsis thaliana* Columbia (Col) had to be characterized. Without light signals, Col seeds are unable to germinate with sufficient oxygen, water and temperature (reviewed by Finch-Savage and Leubner-Metzger, 2006) even after cold stratification and months of after-ripening. Nitrate alone was a sufficient signal to initiate dark germination (Fig 3.3.1).

Closer inspection of nitrate concentration response showed that Col had a dual peak response to high (20 mM KNO_3) and low (5 mM KNO_3) concentrations (Fig 3.3.2). This relates to the work of Alboresi *et al.* (2005) who found that higher levels of seed stored NO_3 reduced dormancy. Furthermore they demonstrated a separate high/low nitrate response by rescuing the germination of the mutant *NITRATE TRANSPORTER 1*(*nrt1.1*) with high nitrate (10 mM), but not low nitrate (1 mM). They found that exogenous nitrate effects were dependent on seed stored nitrate levels.

Nitrate signaling stimulating dark germination was broken in the *wox7* mutant (Fig 3.3.3). However the mutant phenotype was curiously rescued by co-application of CK, although CK alone had no effect. Cytokinin also enhanced the wild type dark germination response in the presence of nitrate (Fig 3.3.3, Fig 3.3.4). This was not a metabolic effect as the null NR activity mutant *nia1/nia2* (Wang R *et al.*, 2004) also responded to nitrate and CK stimulation of dark germination (Fig 3.3.10). This effect was weak at the low nitrate peak, but pronounced at the high nitrate peak. However, both nitrate levels showed a high (3 μM)/low (0.5 μM) peak response to cytokinin application, even though high exogenous cytokinin inhibits light induced germination (Fig 3.3.1). Only the high level combinations of cytokinin and nitrate rescued the *wox7* phenotype (Fig 3.3.5) and the effectiveness seemed to decrease over storage even though the same *wox7* seeds germinated 100% under continuous light.

An explanation of the observed cytokinin effects based on current literature is difficult. Wang *et al* (2011) observed that cytokinin down-regulated *ABI5* expression, which in-part mediates ABA suppression of germination (reviewed by Finch-Savage and Leubner-Metzger, 2006). On the other hand, Riefler *et al.* (2006) observed increasing levels of dark germination in higher order cytokinin receptor (AHK/CRE1) mutants on high nitrate containing MS media. However their data suggests faster germination under light, which does not match with the finding that cytokinin activated ARR4 stabilizes PHYB red light signaling activity (Sweere *et al*., 2001, Mira-Rodado *et al.*, 2007).

The *phyB* mutant germination phenotypes (Fig 3.3.8) resemble *wox7*. Dark germination was near 0% in the Col *phyB* mutant and 100% under continuous white light matching what Shinomura *et al* (1994) found for the Ler *phyB* mutant. Lee *et al* (2012) showed that removing the aleurone of *phyB* seed embryos allowed full germination, which is also true for aleurone free *wox7* seed embryos. Dark germination of *wox7* seeds was fully recovered by GA, which also rescues *phyB* (Yang *et al.*, 1995). PHYB is required for short pulse red and green light germination stimulation (Fig 3.3.8) and *wox7* is more resistant then the wild type to short light pulses (Fig 3.3.8). This suggests that the *WOX7* germination effect may work through PHYB activity, so the relationship of *PHYB* to nitrate and CK was examined.

Nitrate and CK co-application fails to stimulate dark germination in the *phyB* mutant (Fig 3.3.9) indicating that dark germination stimulation by nitrate/cytokinin signaling is upstream of PHYB activity. As PHYB acts by degrading PIF1 (Shen *et al.*, 2008) leading to eventually lowered ABA and increased GA levels (Reviewed by Rajjou *et al.*, 2012), it is not a surprise that nitrate and CK does not rescue the GA mutant *ga1-3* (Fig 3.3.11).

To figure out the mechanism behind the *wox7* phenotype, the *WOX7* expression pattern was examined with a pWOX7::GUS mutant from the SAIL line (Sessions *et al.*, 2002). No pWOX7 expression was detected during dark germination (Fig 3.3.7) nor was there any *WOX7* transcript detected with reverse transcriptase PCR at 0, 24 or 48 h post stratification (Supplemental Figure 3.A,1).

During seed development (Fig 3.3.6) pWOX7::GUS is seen in the germinated pollen and the micropylar end of the embryo sac. Ubiquitous expression in developing seeds ends after embryo development passes through the heart/torpedo stage; sporadic expression was observed in a few cotyledon stage embryos in the hypocotyls and cotyledons. Expression of pWOX7::GUS appears post germination during high nitrate or high cytokinin induced cotyledon opening (Fig 3.3.7) in the SAM, cotyledons and petioles. Cytokinin induced photomorphic responses such as cotyledon opening during dark germination have been observed before (Chin-Atkins *et al.*, 1996). High nitrate caused similar effects, which are more pronounced when the seed coat is removed (Supplemental Figure 3.B.1).

The coat effect may be related to aleurone released ABA (Lee *et al.*, 2010), which cytokinin antagonizes during germination (Wang *et al.*, 2011). Cytokinin induced dark cotyledon opening has been linked to *WOX9* (Skylar and Wu, 2010), however their method included an 8 hour light incubation after stratification, which may explain the difference in their results. The *wox7* mutant does differ in cotyledon opening from the wild type, which suggests *WOX7* is only an accessory transcribed in response to cytokinin activity.

Considering that cytokinin has been shown to act through PHYB in an ARR4 protein-protein interaction (Sweere *et al.*, 2001, Mira-Rodado *et al.*, 2007), it is possible that the *wox7* phenotype is due to lowered levels of seed stored PHYB. High levels of pWOX7 expression during seed development was found during the early stages, which may suggest either that early development may affect seed storage or low levels of *WOX7* expressed in specific tissues during later stages was below detection. High cytokinin may increase the stability of Pfr PHYB (Sweere *et al.*, 2001), which may be activated in a nitrate dependent pathway. These hypotheses can be evaluated in future works.

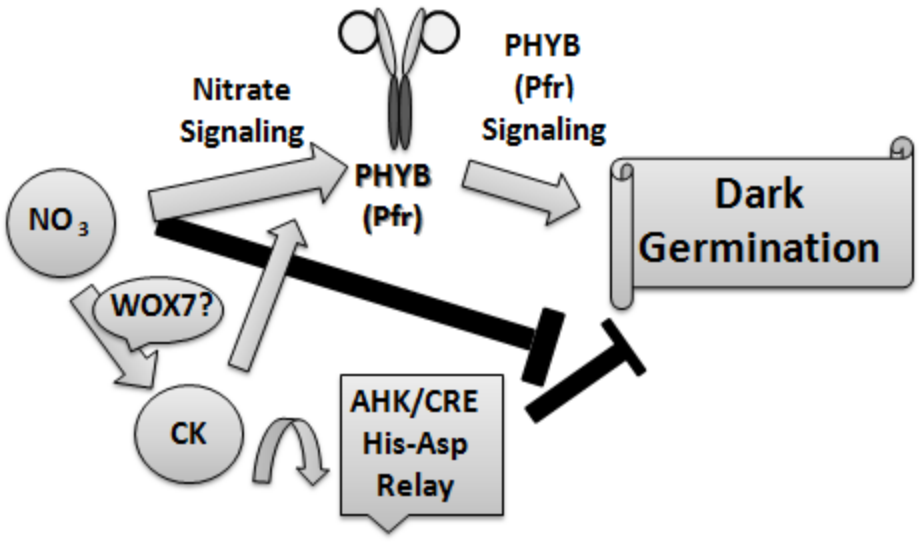

Fig 3.4.1 Nitrate Induced Dark Germination Model

Nitrate acts through PHYB to induce germination under darkness. Nitrate induction of dark germination is enhanced by the presence of CK. Cytokinin suppresses germination when applied alone, which is relieved by loss of AHK/CRE CK receptors that signal through the His-Asp relay. Therefore nitrate signaling must overcome CK repression. Nitrate induction is lost in *wox7*, but restored by CK, suggesting that WOX7 is involved in proper endogenous CK response to nitrate.

The results of this chapter suggest that dark germination in Arabidopsis Columbia requires nitrate signaling. High concentration nitrate signaling is enhanced by cytokinin co-application and act upstream of PHYB, likely to activate it (Fig 3.4.1). Proper PHYB activation by nitrate requires WOX7 activity (putatively) during seed formation as *WOX7* was not detected during dark germination. Loss of *WOX7* can be rescued by high nitrate and high cytokinin co-application. This suggests that the loss of dark germination in *wox7* may be due to lowered production and/or sensitivity to a nitrate signaling intermediate that is up-regulated by cytokinin, possibly nitric oxide, which is rapidly induced by cytokinin. Future work should focus on discovering potential candidates.

3.5 Materials and Methods

Plant Material and Growth Conditions

All *Arabidopsis thaliana* plants used were Columbia unless otherwise specified. Wild types were Col-0. SAIL (Sessions *et al.*, 2002), SALK (Alonso *et al.*, 2003) T-DNA insertion lines (Col-3 and Col-0 backgrounds respectively) and other mutant lines were obtained from the Arabidopsis Biological Resource Center. Homozygous progenitors were screened by PCR (Supplemental Table 4.A). Primers and insertion locations were obtained from Signal Salk (http://signal.salk.edu/). The *nia1/nia2* mutant (Wang R *et al.*, 2004) was verified by death of 50 seedlings grown with nitrate as the sole source of nitrogen. The phytochrome mutants were qualitatively verified for phenotypes previously demonstrated in literature (reviewed by Franklin and Quail, 2010); *phyA* mutants was selected for long hypocotyls under far-red light; *phyB* and *phyD* were selected for long hypocotyls under red light. Seeds were surface sterilized with 75% ethanol and 0.1% Triton-X for 10 min, followed by a 95% ethanol wash for 1 min. Seeds were dried on sterile filter paper before being sown on solid media (½Murashige Skroog (MS) [plantMedia], 1% sucrose, 0.3% Phytagel [Sigma®]). To synchronize the seeds they were stratified at 4^{o}C for 3 days. Plates were germinated under 24 h light (warm white fluorescent [Philips] at 50±5 umol s^{-1} m^{-2}) and kept for two weeks at 22±2^{o}C. Seedlings were transferred into soil (Sunshine LA4 aggregate plus [Sungro®]) pots with individual plants separated by plastic dividers (Arasystem [Betatech]). Potted plants were grown in a growth chamber (16h/8h day night cycle, warm white fluorescent at 50±5 umol s^{-1} m^{-2}, 22^{o}/18^{o}C) and watered with tap water twice a week.

DNA Extraction

Explants were homogenized in salt extraction buffer (1.25 mM EDTA, 40 mM TRIS, 12.5 mM NaCl, 0.025% SDS) and then mixed with chloroform. The mix was separated by centrifugation (15k g, 5 min) and the top aqueous layer was transferred. DNA was precipitated out by adding one volume of isopropanol and centrifugation (15k g, 5 min). Supernatant was discarded and the pellet was air dried. Dry pellets were dissolved in diethylpyrocarbonate (DEPC) treated water and boiled to evaporate residual isopropanol and denature any DNAse contaminants (96^{o}C, 10 mins) before storage at -20^{o}C.

PCR

PCR was carried out using a (1:2:6:6) mixture of DNA template, primer, water and BioMix [Bioline®]. Confirmation reactions for SALK lines were run using the following cycle; 94^{0}C (5 min) {94^{o}C (30 s): 60^{o}C (30s): 72^{o}C (45s)} x36 cycles. SAIL & GABI-KAT lines were run with a 56^{o}C annealing temperature.

Germination Tests

Dark Germination: Seeds were surface sterilized with 75% ethanol and 0.1% Triton-X for 10 mins, washed with 95% ethanol for 1 min and then washed 3 times with water. Sterilized seeds were then plated on 0.8% agar, deionized water plates (plus treatments where indicated), 50 per plate, under cool white florescent lighting (15±3 umol s^{-1} m^{-2}). Each plate was sealed with parafilm and replicate groups of 3 were wrapped in aluminum foil. They were then dark stratified at 4^{o}C for 3 days. After stratification the plates were transferred into a dark growth chamber (22±2^{o}C) until counting. Counts were scored by radicle emergence. Each experiment was trialed once.

Light Treatments: Post stratification, plates were subject to light treatments (30±2 umol s^{-1} m^{-2}) as specified inside a dark room (22±3^{o}C). The lights used were as follows: 740 nm Far Red [LedEngin LZ4], 660 nm Deep Red [LedEngin LZ4], 540 nm Green [LedEngin LZ4], 450 nm Royal Blue [Cree® XTE]

Seed Dissection: Seeds were surface sterilized, plated on wet filter paper and kept at 4^{o}C overnight. Seed coats were removed by microsurgery and dissected embryos were plated as specified in treatments on 0.8% water agar. Dissections occurred under cool white fluorescent lighting (30±3 umol s^{-1} m^{-2}). Plates were then stratified as per dark dissections.

Microscopy and GUS Staining

GUS staining was done as described previously (Weigel and Glazebrook 2002). Explants were fixed with 90% acetone for 15 min at 4^{o}C. Explants were then incubated with GUS staining solution (1 mM EDTA, 5 mM $KFe(CN)_6^{-4}$, 5 mM $KFe(CN)_6^{-3}$, 100 mM $NaPO_4$, 1% Triton-X, 2 mM X-Gluc) at 37^{o}C for 4 hours, except pWOX7 detection during dark germination which was 24 hours. Microscope pictures were taken with a 420 DFC [Leica] attached to a Wild M8 dissecting microscope [Leica].

Statistical analysis

All statistical analysis was performed with one-way ANOVA tests. SigmaPlot© [Systat®] was used for calculations.

GENERAL DISCUSSION AND CONCLUSIONS

Somatic Embryogenesis Phenotype of *wox* Mutants

This chapter was abandoned without closure due to the low viability of completion within the allotted time frame. Most of the *WUSCHEL HOMEBOX* (*wox*) mutants were not significantly different from the wild type in somatic embryogenesis (SE) response. Excluding *wox2* and *wox9*, which were not isolated, *wox8* and *wox12* had reduced SE, while *wox13* had increased SE response. Pursuit of the *wox13* phenotype was not possible due to late identification of the mutant and lack of readily available resources, such as over-expression mutants or reporter lines. Therefore the work was focused on the earliest identified mutant with an available GUS line, *wox7*. Although *wox7* was not statistically significant in SE enhancement, responded consistently higher over three separate trials and its expression pattern was consistent with an inhibitor suggesting biological significance.

WOX7 was expressed transiently under light induction where SE response was high, but constitutively expressed during dark induction where SE was repressed. Expression of *WOX7* was found in embryogenic tissue, shoot regions where callus would form and within formed callus tissue. Although *WOX7*'s identity as a SE inhibitor is only speculative, some insight can be gained by considering the expression pattern. Callus that retained high *WOX7* expression did not differentiate further. Expression of SE competence marker *SERK1* (Supplemental 2.B.1) was lower while *WOX7* was highly expressed (up to day 6).

Unfortunately the wox7 mutant did not respond differently from the wild type to common vegetative hormone response tests for auxin, CK, ABA and GA, although that work did uncover the *wox7* dark germination phenotype covered in chapter 3. Without any evidence suggesting how WOX7 worked and an utter lack of literature the *wox* analysis was abandoned and the focus was switched to elucidation of the reason of why light induction produced a higher SE response then darkness.

Light Induction of Somatic Embryogenesis

This intention of this chapter was to test whether a hypothetical model for light entry into the SE pathway was valid. The model (Fig C.1) was derived from approaching the question from two opposite points; the effect of light and the *AGL15* transcription factor.

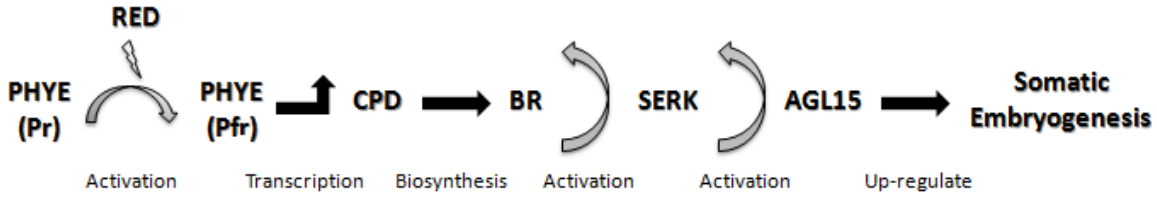

Fig C.1 Light Induction of Somatic Embryogenesis Model
Hypothetical model of light induction of somatic embryogenesis. Red light activates PHYE leading to increased transcription of *CONSTITUTIVE PHOTOMORPHOGENIC DWARF* (*CPD)*. Increased CPD increases brassinosteroid (BR) biosynthesis. BR reception activates SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 1 & 5 (SERK1/5) which activates AGAMOUS LIKE 15 (AGL15). AGL15 activates SE response in competent cells.

Light as a plausible SE entry signal was fairly well represented in literature albeit in other species. *AGL15* was a far downstream SE factor that had been validated independently by different research teams. Light induction and specifically red light (660 nm) was confirmed to be a SE entry signal in Arabidopsis Columbia. AGL15 and protein complex associated SERK1 were reconfirmed by knockdown mutant analysis to be necessary (but not necessarily sufficient) for SE. Bridging these two points was BR.

Red light reception through PHYE was shown to be necessary for up-regulation of the BR biosynthesis rate limiting enzyme CPD. *CPD* regulation by PHYE was not instantaneous, appearing at day 5 and not day 3 of light induction. This suggests that either the explants were not competent for BR signalling at day 3 or PHYE levels were not sufficient at that time or that PHYE indirectly regulated *CPD*. Lower levels of CPD transcription was present under darkness and in phyE, which could explain the low levels of background SE. Given that previous studies have shown that BRs may improve SE

response, the litmus test is whether BR rescues the reduction of SE by darkness or *phyE*. From the red light to PHYE entry point the following is suggested for continued research:

1. Is PHYE the only phytochrome controlling SE?

   As no *phyC* mutant was isolated within the project timeframe, the obvious test is to examine *phyC* SE response. As SE reduction by darkness is greater than *phyE* it is possible that *phyC* also controls SE.
2. Is PHYE response monomeric or due to heterodimerization?

   PHYE forms an obligate heterodimer only with PHYB and PHYD. It is possible to assess whether the SE response is monomeric by examining the SE response of a *phyB/phyD* double mutant. If *phyB/phyD* does not pheno-mimic *phyE* then the response is monomeric. The SE response of PHYE is likely monomeric as *phyB* is similar to wild type and *phyD* has improved SE.
3. What is the PHYE level during SE induction?

   A basic characterization of PHYE protein levels during SE can close off many possibilities by Western Blotting different time points during SE with a PHYE antibody. *PHYE* has stable transcription and is high in the reproductive tissue, so fluctuation is likely to be low.
4. Does PHYE directly or indirectly control *CPD* transcription?

   A protein synthesis inhibitor like cyclohexamide could be used to examine if PHYE can directly change CPD levels without *de novo* protein synthesis.
5. Is BR signalling the step following red light response?

   The simplest test is to check whether BR application rescues either darkness or *phyE*. If the result is positive then the answer is obvious. However a negative result is not obvious, as downstream SE components may also be directly regulated by light.
6. Which BR receptor(s) handles SE initiation?

   The BR receptor *bri1* does not affect SE response level. There are three other known receptors *BRL1, 2, 3*. The brute force method for examining this would be to check each mutant and then higher order mutants. The problem with higher order BR mutants is sterility, so if the receptors are redundant this may not be resolvable directly.

The suggested experiments would first determine if PHYE is the sole transducer of red light signals to SE and whether its activity was monomeric. PHYE downstream activity connecting to *CPD* needs to be established. Finally increases in *AGL15* transcription due to BR needs to be directly connected to SE.

As the *bril* mutant was not significantly different in SE response, BR could not be directly connected to SE. However model (Fig C.2) depicts a testable hypothetical BR interaction through *SERK1/5* based on past literature. SERK1 and 5 have cytoplasmic kinase domains, but do not have extracellular receptor domains so they rely on dimerizing with true receptors. Dimerization of SERKs with BRI1 has been demonstrated as well as dimerization/interaction with over 20 other RLKs by SERK3. RLK trans-phosphorylation has been demonstrated in BRI1 and this probably also applies to the BRLs. Activation of SERK1/5 by (a) BR receptor(s) by trans-phosphorylation is possible.

Activated SERK1/5 could activate AGL15 in the following manner. AGL15 is a DNA-binding transcription factor; therefore the observation that it is found in the cytoplasm as well as the nucleus suggests a cytoplasmic sequestering control method. Coincidently proteins with a known mechanism of cytoplasmic sequestering, 14-3-3 proteins, have been found in a protein complex with SERK1 and AGL15 at the plasma membrane. 14-3-3 proteins have been shown to bind to DPB, BZR1 and BZR2 and retain them in the cytoplasm. Coincidently 14-3-3 shuttling is controlled by phosphorylation. Thus it is possible that AGL15 is sequestered in the cytoplasm by 14-3-3 proteins until interaction with activated SERK1/5 disengages the 14-3-3 and allows nuclear import of AGL15. Now this is all completely speculative, but the beauty of it is that all of the above can be validated (or refuted) through experimentation.

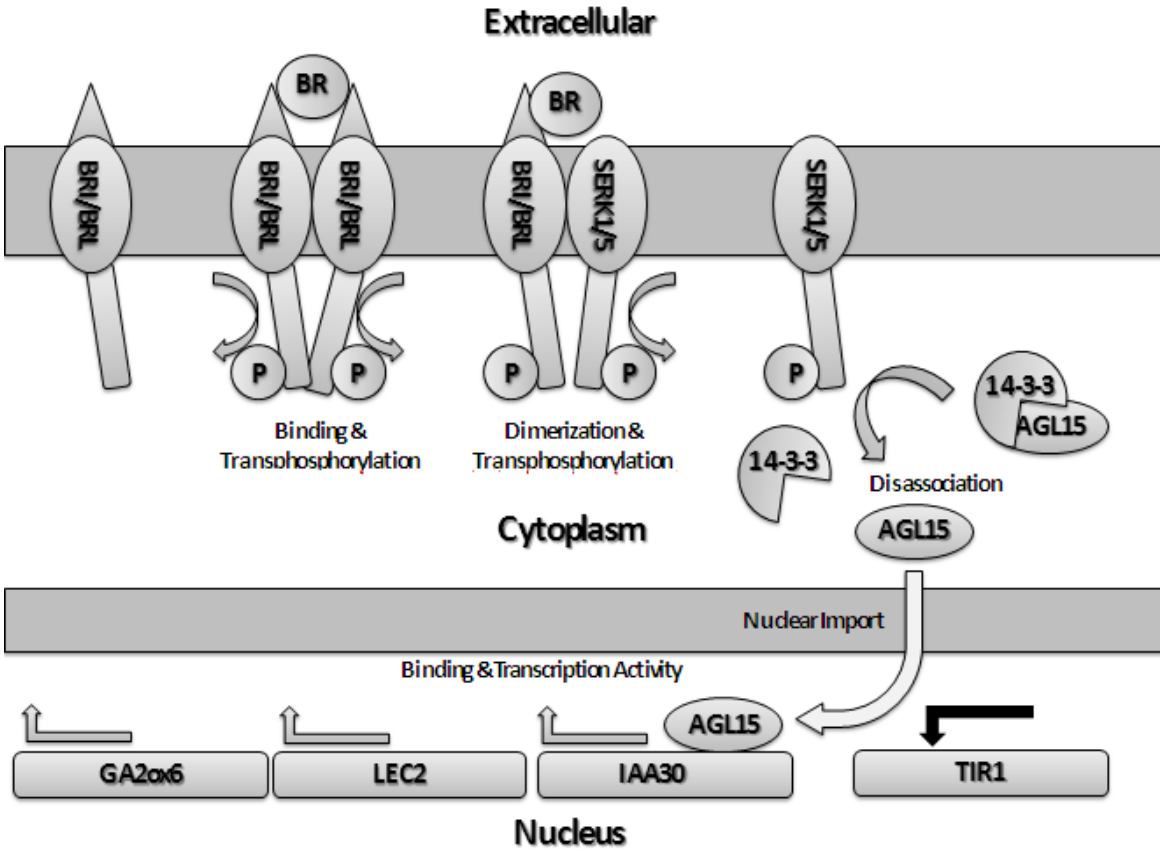

Fig C.2 Hypothetical Model of BR Reception to AGL15 mediated SE

Hypothetical model of how BR reception could lead to somatic embryogenesis response in a SE competent cell based on previous literature. BRI1 LIKE (BRL) receptor-like kinases (RLK) dimerize and activate by transphosphorylation upon reception of BR. Activated BR RLK dimerizes with SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 1/5 (SERK1/5) (or removes an inhibiting dimer partner) and activates SERK1/5 presumably through kinase activity. Activated SERK1/5 disassociates 14-3-3 proteins from AGAMOUS LIKE 15 (AGL15). AGL15 nuclear imports and binds to the promoters of *GA2 OXIDASE 6* (*GA2ox6*), *LEAFY COTYLEDON 2* (*LEC2*) and *INDOLE-3-ACETIC ACID INDUCIBLE 30* (*IAA30*) resulting in up-regulation of transcription, while down regulating *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*). Transcriptional activity eventually leads to SE formation.

1. Does the nuclear localization of AGL15 determine SE response?

   Examining whether modified AGL15 insertions rescue the SE phenotype of *agl15* can demonstrate domains required for activity. Of interest is removing the DNA-binding motif(s) or the nuclear import domain, assuming they could be identified. Confirmation of transcriptional activity could be achieved by a promoter with an AGL15 binding site driving a marker like a fluorescent protein (FP).

2. Which 14-3-3 proteins bind AGL15?

   A protein complex immune precipitation (Co-IP) or yeast two hybrid (Y2H) assay would identify candidate 14-3-3s. A *14-3-3* mutant could possibly enhance SE due to uncontrolled AGL15 activity (confirmable by *agl15/14-3-3* being like *agl15*), although since 14-3-3s chaperone many proteins the effect may be cloudy.

3. Do 14-3-3s inactivate AGL15 by cytoplasmic sequestering?

   If ectopic *14-3-3* expression suppresses wild type SE and antagonizes SE enhancement by ectopic *AGL15,* then that should confirm the effect of 14-3-3 on AGL15. Examination of sub cellular localization of FP-tagged AGL15, 14-3-3 and AGL15+14-3-3 (bimolecular fluorescence complementation (BiFC)) should show that free AGL15 is mostly in the nucleus while the AGL15/14-3-3 complex is mostly in the cytoplasm (mostly as there should be in-transit proteins). Finally the methods used in past experiments to examine 14-3-3 sequestering of DBP1, BRZ1 and BZR2 should be used.

4. Does SERK1/5 release AGL15 from 14-3-3 sequestering?

   If SERK1/5 releases AGL15 then the *serk1/5* double mutant should have reduced nuclear localized FP-tagged AGL15 or increased cytoplasmic BiFC of 14-3-3+AGL15. The phosphorylation states of 14-3-3 and AGL15 can be assayed using protein mobility shifts as per the DBP1 experiments.

5. Is BR response dependent on SERK1/5 and/or AGL15?

   If BR application fails to have any effect on the *agl15* mutant or *serk1/5* double mutants then BR response is dependent on SERK1/5 and AGL15.

6. Do BRs activate SERK1/5 to release AGL15?

   This is probably the trickiest part. If the BR receptors are highly redundant then verification using BR antagonists/inhibitors will be difficult. Otherwise the pharmacological effect or BR receptor mutants related to SE should result in at least a partial *serk1/5* phenotype.

First it must be proved that AGL15 requires nuclear localization for activity to consider hypothetical protein chaperone based control by 14-3-3. If 14-3-3 sequestering does modulate AGL15 activity, then a 3-FP system (free AGL15 [cyan], free 14-3-3 [red], AGL15/14-3-3 complex [purple] and AGL15 transcription marker [yellow]) could clearly show what outcomes are caused by the background. If the background mechanism that releases AGL15 from 14-3-3 were to be removed, then AGL15 transcription activity should be minimized (residual due to partial redundancy by AGL18). Furthermore AGL15/14-3-3 complexes would be predominant (entropy would free a small fraction) and ideally this would be the case in the *serk1/5* or BR suppressed backgrounds.

Excluding the unproven BR to SERK to AGL15 link, this project was able to demonstrate both that light was a SE inducing signal in Arabidopsis immature zygotic explants and that light had a clear mechanism. Red light activated PHYE up-regulated BR biosynthesis which up-regulated *AGL15* transcription. This sequence of events was not required for callus formation or survival, specifying it as a requirement for SE. Future work will build on extending the chain and elucidating the underlying principles of action.

Dark Germination Phenotype of *wox7*

Attempting to elucidate the *wox7* dark germination phenotype required characterizing the basic requirements of Arabidopsis Columbia (Col) dark germination as previous literature lacked standardization.

Dormancy is reduced in Col by after-ripening and cold stratification is necessary for efficient dark germination. After stratification only the presence of nitrate allowed Col to germinate in the dark. Dark germination response to nitrate exhibited high (20 mM) and low (~5 mM) peaks. Past literature suggests that nitrate signalling is separated between high and low concentration receptors (perhaps even pathways), with low nitrate mediated by the dual-affinity nitrate transporter 1 (NRT1.1). Nitrate metabolism was unnecessary as nitrate reductase (*nr*) null mutants responded to nitrate dark germination stimulation. Nitrate signalling (as opposed to metabolism) is not well elucidated so a working hypothesis was not proposed.

The high concentration response was more useful due to its synergy with cytokinin (CK) and was used for experiments. These concentrations are likely not absolute as the dormancy of seeds has been shown to be linked to stored nitrate, which is primarily controlled by maternal nitrate levels. The mother plant was grown in soil so the nitrate content wasn't known. Any retrials using different growth conditions should rebuild a standard nitrate response ladder for the first batch of seeds. The seeds in these tests were sown under ambient light and kept in darkness during cold stratification until counting.

Ambient light prior to imbibition did not have obvious effects. Post stratification short light pulses (15 min) strongly affected subsequent high nitrate induced "dark germination". Red (660 nm) and green (540 nm) light allowed complete germination, while blue (470 nm) and far red (740 nm) repressed nitrate induced germination. Red or green light pulse (but not continuous) and nitrate induced dark germination requires functional PHYB.

PHYB is currently the earliest entry point known in dark germination. The results now show that nitrate and cytokinin act upstream of PHYB as neither nitrate nor nitrate supplemented with CK can cause *phyB* or the downstream gibberllic acid (GA) mutant *ga1-3* to germinate. CK has established crosstalk with *PHYB* through *ARABIDOPSIS RESPONSE REGULATOR 4* (*ARR4*).

Previous studies have shown that ARR4 binds to PHYB. Furthermore CK induced phosphorylation of ARR4 maintains the Pfr form of PHYB, increasing its red light response activity. One study showed that AHK/CRE CK receptor mutants have higher dark germination, which suggests that the two component His-Asp phospho-relay system is not the pathway through which CK affects dark germination through. This is because this thesis' results clearly demonstrate that high nitrate dark germination is enhanced or suppressed by CK in a concentration dependent fashion.

A high (3 μM)/low (0.5 μM) peak pair is observed for response to CK application. The dual peak was unexpected and the high CK peak may be an *in vitro* artifact. CK alone cannot induce dark germination and retards *PHYA* and *PHYE* mediated continuous light germination, as seen by the CK repression of *phyb* light germination. Given past works showing CK biosynthesis induction by high nitrate in roots and feedback signalling, there is reason to believe that endogenous CK signalling is a subordinate pathway to high nitrate signalling. The low nitrate response has similar CK peaks, but no significant increase in germination due to CK. Cytokinin has been shown to cause photomorphogenic effects such as dark cotyledon opening. This CK phenotype was mimicked by high nitrate application, further evidence that high nitrate signalling involves CK. Most importantly CK co-application with high nitrate rescues the *wox7* dark germination phenotype.

The *wox7* seeds germinate 100% under light but basically do not dark germinate. This phenotype mimics *phyB*. Like *phyB* short red or green light pulses (15 min) fail to cause germination in *wox7*. Long pulses (1 hr) resulted in moderate dark germination.

This suggests that *wox7* may have weaker PHYB activity, although whether this is due to lower PHYB levels or PHYB accessories is yet to be identified. It is unlikely that WOX7 directly interacts with the PHYB pathway during dark germination as *WOX7* expression was not detectable during dark germination. Thus it is likely that the *wox7* phenotype arises from effects carried forward from seed setting, where *WOX7* is expressed in the early chalazal region of the embryo sac.

As with previous literature the aleurone layer, where PHYB mediated germination occurs, was responsible for active suppression of germination in the *wox7* mutant. Aleurone-free seed embryos and seeds with lacerated coats of *wox7* germinate as readily as the Col-0 wild type, without need for exogenous nitrate or CK application. However the suppression of growth by seed coat bedding was not observed with *wox7* coats. This was previously shown to be related to abscisic acid (ABA) synthesis and release by the aleurone in dormant seeds.

When wild type seeds were sown on the same media as *wox7* seeds, their dark germination was slightly delayed, suggesting that *wox7* releases a low amount of ABA. ABA sensitivity under light germination is NSD between the wild type and *wox7*. Therefore germination suppression in *wox7* is probably not due to hyper ABA response. Transcripts for *ABSCISIC ACID OXIDASE 3(AAO3),* vital for ABA synthesis for dormancy, in *wox7* seeds 48 hours after stratification is not higher then wild type and the ABA catabolism gene *CYP707A2* was actually found to be greater than Col-0.

The ABA activated germination suppressor *ABSCISIC ACID INSENSTIVE 5* (*ABI5*) had higher transcription in *wox7*. Not surprisingly the ABI5 inhibitor, GA, had lower biosynthesis transcription (*GAox1, 2*) then wild type. GA at low levels (0.1 μM) rescues *wox7*. The physiological state of the *wox7* seed is likely to be low ABA and low GA under conditions where the wild type can germinate.

The last clue to why *wox7* does not germinate in the dark is the fact that the phenotype can be completely rescued by high CK (3 μM) co-applied with high nitrate (20 mM). This rescue applied even after months of after-ripening which contrary to the wild type actually lowers *wox7* dark germination. Interestingly the low CK (0.5 μM) peak does not rescue *wox7*, suggesting that the high CK response may over produce a nitrate signalling intermediate, possibly nitric oxide. This also suggests that *wox7* is not likely to be simply impaired in CK synthesis in response to nitrate as there should've been a CK concentration response shift if this were the case.

As available literature concerning both nitrate signalling during germination and dark germination is poor and non-standardized, only a crude model could be produced (Fig C.3).

One underlying assumption based on past literature is that endogenous CK levels are low (usually reported at pmol g^{-1} levels) although it is unknown what the *effective perceived CK level is*. If endogenous CK during dark germination is low then the nitrate mediated low CK response, which does not rescue *wox7*, should be in effect. Thus it is possible that WOX7 is involved in preparing the maturing seed to be capable of responding to low CK during nitrate induced dark germination. Nitrate in concert with CK would activate endosperm PHYB Pfr activity which would lead to GA activity eventually overcoming ABA activity and releasing dormancy. To prove this of course would require alot of work.

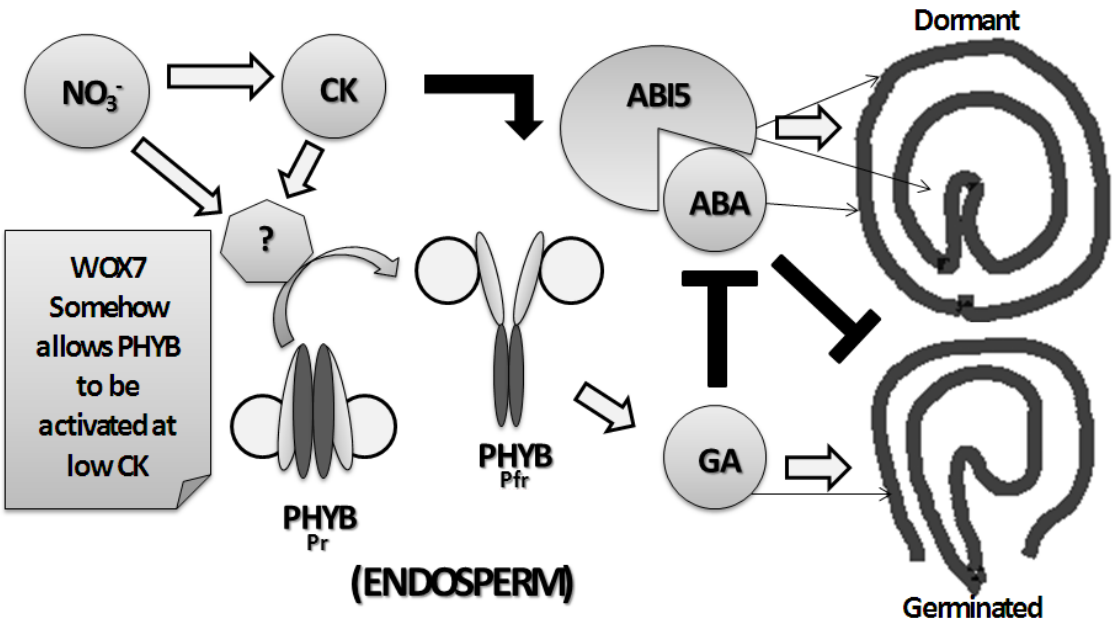

Fig C.3 Nitrate Induced Dark Germination Model

Nitrate signalling and CK likely shifts PHYB to the Pfr conformation; as far-red light, which shifts PHYB to Pr, reduces nitrate induction of dark germination. Based on previous literature, Pfr activity leads to increased GA that suppresses aleurone released ABA. ABA activated germination suppressor ABA INSENSTIVE 5 (ABI5) is down-regulated by CK. Endogenous CK response is activated by WOX7 activity to shift PHYB to Pfr.

The state of dark germination research is poor. As such further elucidation of the wox7 phenotype can only be indirectly approached. These are some preliminary tests to examine how the *wox7* phenotype arises.

1. Is the *wox7* phenotype maternal?
   By examining the dark germination phenotype of reciprocal crosses between Col-0 and *wox7*, it can be determined whether the effect is maternal..
2. Is the *wox7* phenotype a result of altered endogenous CK or CK perception?
   To validate whether CK sensitivity during dark germination is perturbed (wox7 does not respond differently from wild type during vegetative growth) the endogenous CK levels during dark germination needs to be measured.
3. Is the wox7 phenotype a result of altered PHYB levels?
   Measuring the PHYB levels in seeds will determine if altered PHYB levels cause the *wox7* phenotype.
4. Is the wox7 phenotype a result of lowered “saved” Pfr activity?
   If *PHYB* over-expression fails to rescue the dark germination phenotype of *wox7* then WOX7 likely is required for seeds to activate Pfr in the dark during nitrate induced germination.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All *Arabidopsis thaliana* plants used were Columbia unless otherwise specified. Wild types were Col-0. SAIL (Sessions *et al.,* 2002), SALK (Alonso *et al.,* 2003) T-DNA insertion lines (Col-3 and Col-0 backgrounds respectively) and other mutant lines were obtained from the Arabidopsis Biological Resource Center. Homozygous progenitors were screened by PCR (Supplemental Table 4.A). Primers and insertion locations were obtained from Signal Salk (http://signal.salk.edu/). The *nia1/nia2* mutant (Wang R *et al.,* 2004) was verified by death of 50 seedlings grown with nitrate as the sole source of nitrogen. The phytochrome mutants were qualitatively verified for phenotypes previously demonstrated in literature (reviewed by Franklin and Quail, 2010); *phyA* mutants was selected for long hypocotyls under far-red light; *phyB* and *phyD* were selected for long hypocotyls under red light. The *bri1* mutant was verified to have a cabbage like vegetative growth and highly reduced fertility (Clouse *et al*., 1996).

Seeds were surface sterilized with 75% ethanol and 0.1% Triton-X for 10 min, followed by a 95% ethanol wash for 1 min. Seeds were dried on sterile filter paper before being sown on solid media (½Murashige Skroog (MS) [plantMedia], 1% sucrose, 0.3% Phytagel [Sigma®]). To synchronize the seeds they were stratified at 4^{o}C for 3 days. Plates were germinated under 24 h light (warm white fluorescent [Philips] at 50±5 umol s^{-1} m^{-2}) and kept for two weeks at 22±2^{o}C. Seedlings were transferred into soil (Sunshine LA4 aggregate plus [Sungro®]) pots with individual plants separated by plastic dividers (Arasystem [Betatech]). Potted plants were grown in a growth chamber (16h/8h day night cycle, warm white fluorescent at 50±5 umol s^{-1} m^{-2}, 22^{o}/18^{o}C) and watered with tap water twice a week.

DNA Extraction

Explants were homogenized in salt extraction buffer (1.25 mM EDTA, 40 mM TRIS, 12.5 mM NaCl, 0.025% SDS) and then mixed with chloroform. The mix was separated by centrifugation (15k g, 5 min) and the top aqueous layer was transferred. DNA was precipitated out by adding one volume of isopropanol and centrifugation (15k g, 5 min). Supernatant was discarded and the pellet was air dried. Dry pellets were dissolved in diethylpyrocarbonate (DEPC) treated water and boiled to evaporate residual isopropanol and denature any DNAse contaminants (96^{o}C, 10 mins) before storage at -20^{o}C.

PCR

PCR was carried out using a (1:2:6:6) mixture of DNA template, primer, water and BioMix [Bioline®]. Confirmation reactions for SALK lines were run using the following cycle; 94^{0}C (5 min) {94^{o}C (30 s): 60^{o}C (30s): 72^{o}C (45s)} x36 cycles. SAIL & GABI-KAT lines were run with a 56^{o}C annealing temperature.

Somatic Embryogenesis Culture Conditions

Green siliques were surface sterilized in 75% ethanol for 5 min. Immature zygotic embryos were obtained by hand dissection of embryo sacs inside the siliques. Late stage embryos (large green cotyledons) were preferred as mortality was higher in younger explants. Explants were kept for 14 days on induction media (Supplemental 4.B) and then transferred to hormone-free, solid media (½MS, 1% sucrose, 0.3% Phytagel [Sigma®]) to mature for 7 days.

When plant growth regulators (PGR) were applied to media, they were applied after auto-claving, except for 2,4-D and BA, which were added prior to auto-claving. PGRs were dissolved in 95% ethanol or filter sterilized water, depending on solubility. Controls for PGR treated media were treated with an equal volume of solvent. Unless otherwise stated induction was under 24 h light (warm white fluorescent at 50±5 umol s^{-1} m^{-2}) and maturation was under 24 h darkness both at 22±2^{o}C. Somatic embryos were scored based on the following criteria: Identifiable, distinct apical and basal regions; independence of epidermis and vascular system from the original explant or other new formed somatic structures. Scoring was done by physically separating every potential embryo by hand and inspection under microscope. Each experiment was trialed once.

Somatic Embryogenesis Light Treatments

Somatic embryogenesis induction plates were subject to light treatments (color: 30±2, white: 50±5 umol s^{-1} m^{-2}) as specified inside a dark room (22±3^{o}C). The lights used were as follows: 660 nm Deep Red [LedEngin LZ4], 740 nm Far Red [LedEngin LZ4], 540 nm Green [Led Engin LZ4], 450 nm Royal Blue [Cree XTE], Warm white [Philips F32TB/TL841]. Darkness was simulated by wrapping plates in aluminum foil.

Promoter Cis-Element Analysis

The -1000 base pair upstream promoter sequences of all *WOX* genes and *WUS* for Arabidopsis Columbia were obtained from The Arabidopsis Information Resource (TAIR). Sequences were parsed for cis-elements using the PLACE tool (Higo *et al.*, 1999) & ATHENA provided p-values for cis-elements when possible (O'Connor *et al.*, 2005). P-value described on the ATHENA website: (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/Tutorial_Statistics.pl).

Hormone Response Assays

Hormone response assays were modified from Weigel and Glazebrook (2002). All hormones were dissolved in 95% ethanol and applied after auto-claving, with controls receiving an equal amount of ethanol. Each experiment was trialed once.

Auxin and Cytokinin inhibition of root growth was assayed by transferring 7 day old seedlings to vertical ½ MS agar plates with 1% sucrose. Plates were kept in the dark at 22±2^{o}C. Five wild type and five *wox7* seedlings were placed in alternation on a plate. 4 replicate plates were used. Auxin was applied as IAA [Sigma®] at 0.1, 0.5, 1, 5 and 10 µM. Cytokinin was applied as 2IP [Sigma®] at 0.001, 0.01, 0.1, 1, 5 and 10 µM.

ABA germination inhibition was assayed by stratifying 3 replicates of 50 seeds on ½ MS + 1% sucrose agar plates for 3 days at 4^{o}C. Plates were then transferred to 24 hours light at 22±2^{o}C. Germination percentages were scored 7 days after transfer. ABA [Sigma®] was applied at 1, 5, 10, 20, 40 µM.

GA dark germination was assayed using 3 replicates of 50 seeds on ½ MS + 1% sucrose agar plates. Plates were kept in the dark at 22±2^{o}C. Germination percentage was scored 7 days after sowing. GA3 [Sigma®] was applied at 0.1, 1 and 10 µM.

RNA Extraction

RNA extractions were modified from the LiCl method described by Onate-Sanchex and Vicente Carbajosa (2008). Polyvinylpyrrolidone-40 (4% $_{w/v}$) was added to the extraction buffer (0.4 M LiCl, 0.2M TRIS, 25mM EDTA, 1% SDS) to protect against phenolics and reduce phenol contamination. Homogenized samples were phase separated by 1:1 volume chloroform and centrifugation (15k g, 5 min). The aqueous layer was re-separated by 1:1 volume of phenol and 2/5 volume of chloroform and centrifugation (15k g, 5 min). RNA was precipitated overnight (-20^{o}C) by increasing LiCl concentration to 2 M. After thawing the solution the RNA was pelleted by centrifugation (15k g, 30 min). After discarding the supernatant the pellet was then dissolved in 500 µl DEPC treated water. Carbohydrates were removed by precipitation (7 µl Na acetate, 250 µl EtOH) with centrifugation (15k g, 10 min). Supernatant was transferred and RNA was ethanol precipitated (50 µl Na acetate, 500 µl EtOH) at -20^{o}C for one hour. RNA was pelleted by centrifugation (15k g, 30 min). After discarding the supernatant the pellet was dissolved in 20 µl DEPC treated water.

RNA extraction for non-seed explants skipped the first chloroform separation step. RNA concentration and quality was determined by nanodrop [Biorad®] after dissolving of recovered RNA in DEPC treated water. Good quality was a value ≥ 2 for the 260/230 ratio and ≥ 1.8 for the 260/280 ratio. DNA was removed from samples using 10 units of DNase I recombinant (Roche®) at 37^{o}C for 1 hour. Dnase was inactivated by boiling (96^{o}C, 15 min) RNA samples were stored at -80^{o}C.

RT-qPCR

Reverse transcriptase reactions were performed following the Reverse Transcription System (Promega®, A3500) protocol for 2 µg of RNA using random hexamers (1 h at 37^{o}C followed by 5 min at 85^{o}C). RT-qPCR was done on a CFX96 (Biorad) with Sso-fast™ Evagreen® (Biorad) using the following cycle; 95^{o}C (2 min), {95^{o}C (3s): 60^{o}C (7s)} x39 cycles. Following the protocol from Biorad each reaction was an 8 µl mix consisting of 4 µl of Evagreen, 1 µl of primer mix and 3 µl of cDNA. All primers were designed to have a Tm at 60^{o}C (Supplemental Table 4.A.2).

Real Time Analysis

All samples were pooled from 150 (day 3 SE induction) or 50 (day 5 SE induction) individuals. Each experiment had three biological replicates. Cycle threshold (Ct) values were taken as the average of three technical replicates and outliers were thrown. All Ct values were normalized to a housekeeping gene (*UBQ10*) using the ΔΔCT method and corrected for primer efficiency (Livak and Schmittgen 2001). Primer efficiencies were calculated from the slope of four 4x serial dilutions for each gene.

Microscopy and GUS Staining

GUS staining was done as described previously (Weigel and Glazebrook 2002). Explants were fixed with 90% acetone for 15 min at 4^{o}C. Explants were then incubated with GUS staining solution (1 mM EDTA, 5 mM $KFe(CN)_6^{-4}$, 5 mM $KFe(CN)_6^{-3}$, 100 mM $NaPO_4$, 1% Triton-X, 2 mM X-Gluc) at 37^{o}C for 4 hours, except pWOX7 detection during dark germination which was 24 hours. Microscope pictures were taken with a 420 DFC [Leica] attached to a Wild M8 dissecting microscope [Leica].

Germination Tests

Dark Germination: Seeds were surface sterilized with 75% ethanol and 0.1% Triton-X for 10 mins, washed with 95% ethanol for 1 min and then washed 3 times with water. Sterilized seeds were then plated on 0.8% agar, deionized water plates (plus treatments where indicated), 50 per plate, under cool white florescent lighting (15±3 umol s^{-1} m^{-2}). Each plate was sealed with parafilm and replicate groups of 3 were wrapped in aluminum foil. They were then dark stratified at 4^{o}C for 3 days. After stratification the plates were transferred into a dark growth chamber (22±2^{o}C) until counting. Counts were scored by radicle emergence. Each experiment was trialed once.

Light Treatments: Post stratification, plates were subject to light treatments (30±2 umol s^{-1} m^{-2}) as specified inside a dark room (22±3^{o}C). The lights used were as follows: 740 nm Far Red [LedEngin LZ4], 660 nm Deep Red [LedEngin LZ4], 540 nm Green [LedEngin LZ4], 450 nm Royal Blue [Cree® XTE]

Seed Dissection: Seeds were surface sterilized, plated on wet filter paper and kept at 4^{o}C overnight. Seed coats were removed by microsurgery and dissected embryos were plated as specified in treatments on 0.8% water agar. Dissections occurred under cool white fluorescent lighting (30±3 umol s^{-1} m^{-2}). Plates were then stratified as per dark dissections.

Statistical analysis

All statistical analysis was performed with one-way ANOVA tests. Somatic embryogenesis data was tested after normalization to the control. Real-time PCR data was tested after ΔΔCT (Livak and Schmittgen 2001) normalization and before logarithmic transformation. Other data was tested without further manipulation. SigmaPlot© [Systat®] was used for calculations.

All chemicals from Sigma® unless otherwise stated.

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SUPPLEMENTAL DATA

Table 1.A.1 Somatic Embryogenesis Response of wox Mutants

The response of *wox* mutants to somatic embryogenesis of *Arabidopsis thaliana* Columbia. Immature, bent cotyledon zygotic explants were under light induction for 14 days followed by 7 days of light maturation. The measured parameters were the percentage of somatic embryo forming callus (%SEFC ± SE), number of somatic embryos per SEFC (SE/SEFC ± SE) and total somatic embryos per explants (Total SE ± SE). Four replicates of 30 explants were used per line. SE = standard error.

	%SEFC ± SE	SE/SEFC ± SE	Total ± SE
WT	59% ± 3%	2.44±0.28	43.00±5.40
wox1	54%± 7%	2.68±0.31	42.70±4.73
wox3	52%± 5%	2.38±0.09	37.00±5.00
wox4	43%± 9%	2.00±0.10	24.75±3.64
wox5	63%± 3%	2.40±0.22	44.00±3.34
wox6	56%± 3%	2.80±0.15	46.33±1.53
wox7	57%± 6%	3.12±0.25	53.00±7.51
wox8	59%± 1%	1.66±0.11	29.64±2.18
wox10/14	55%± 3%	2.08±0.15	34.41±2.43
wox11	37%± 7%	1.76±0.04	19.67±4.01
wox12	24%± 2%	1.49±0.19	10.58±0.59
wox13	55%± 9%	4.79±0.11	78.09±13.45

Table 1.B.1 Transcription Factor Binding Sites Found in WOX Promoters

Putative transcription factor binding site consensus sequences were identified in the -1000 bp promoter regions of WUS (0) and WOX1-14 using PLACE (Higo *et al.*, 1999) & ATHENA (O'Connor *et al*., 2005). P-values from ATHENA (O'Connor *et al*., 2005). Right column shows the *WOX* members whose promoters contain the element and the number of cis elements contained is shown in the brackets.

Sequence	**p-value**	**Cis-element**	**Contained in WOX** (# of motif)
BACGTGKM	**0.7889**	**ABRE-like**	**4** (2), **13** (1)
TTWCCW$_4$NNGGWW	**0.1496**	**AGAMOUS**	**13** (1)
TTAATGG		**AG** (WUS intron)	**2** (1), **9** (1)
CW$_8$G	**0.0085**	**AGL15**	**1** (3), **2** (1), **3** (4), **4** (2), **5** (5), **6** (2), **7** (1), **8** (1), **9** (2), **11** (3), **12** (3), **13** (2), **14** (2)
AGATCCAA		**AGP1**	**2** (1), **5** (1)
ACTCAT		**B2**	**0** (1), **3** (2), **6** (2), **7** (1), **8** (1), **12** (2)
TAATMATTA	**0.4490**	**HB2**	**12** (1), **14** (1)
CAATTATTA	**0.4106**	**HB6**	**0** (1)
TGTCA		**BELL**	**0** (3), **1** (3), **2** (2), **3** (1), **5** (4), **6** (2), **9** (4), **10** (2), **12** (3), **14** (2)
GGTTAA	**0.6899**	**BOXII**	**1** (1), **8** (1), **11** (1), **12** (2), **13** (2)
ACACNNG		**DPBF**	**0** (2), **4** (1), **5** (6), **7** (1), **11** (1), **12** (2), **13** (1), **14** (1)
WTTSSCSS		**EF2-DP**	**2** (2), **6** (1)
CACGTG	**0.8855**	**GBF4**	**12** (1), **13** (2)
KWGTGRWAAWRW		**GT-1**	**1** (1), **3** (1), **7** (1)
CCAATGT	**0.3560**	**LEAFY**	**2** (1), **12** (1), **14** (2)
CATGCA		**LEC2**	**0** (1), **1** (2), **2** (1), **3** (1), **4** (2), **5** (2), **7** (1), **9** (1), **11** (1), **12** (1)
CACCTG	**0.7701**	**RAV1**	**0** (1)
ACTGTGTA		**SPBF**	**14** (1)
TACTATT		**SPBF**	**4** (1), **5** (1)

Table 1.B.2 Temporal Spatial Expression Cis-Elements Found in WOX Promoters
Sequences correlated to location and timing specific expression were identified in the -1000 bp promoter regions of WUS (0) and WOX1-14 using PLACE (Higo *et al.*, 1999) & ATHENA (O'Connor *et al*., 2005). P-values from ATHENA (O'Connor *et al*., 2005). Right column shows the *WOX* members whose promoters contain the element and the number of cis elements contained is shown in the brackets.

Sequence	**p-value**	**Cis-element**	**Contained in WOX** (Repeats)
$CACGA_4$		**Cell cycle** expression	**0** (1)
$CAAN_4ATC$		**Circadian** expression	**1** (4), **3** (2), **4** (2), **6** (2), **7** (1), **9** (1), **10** (1), **14** (1)
AACAAAC		**Endosperm** expression	**0** (1), **1** (1), **2** (1)
GTACGTG			**3** (2)
TGAGTCA			**0** (1), **3** (1), **13** (1)
TAAATGYA	**0.6070**	**L1** expression	**1** (1), **5** (1), **8** (1)
AGAAA		**Pollen** expression	**0** (12), **1** (6), **2** (8), **3** (13), **4** (9), **5** (8), **6** (9), **7** (7), **8** (14), **9** (8), **10** (5), **11** (1), **12** (7), **13** (8), **14** (8)
GTGA			**0** (11), **1** (12), **2** (5), **3** (8), **4** (9), **5** (4), **6** (5), **7** (10), **8** (5), **9** (10), **10** (4), **11** (5), **12** (3), **13** (3), **14** (10)
AAACCCTAA	**0.7334**	**Root primordia** expression	**7** (1)
CAAACAC		**Seed storage protein**	**7** (1), **11** (1)
$CCN_{12}CCACG$	**0.0711**	**Unfolded Protein Reponse**	**2** (1), **13** (1)

Table 1.A.3 Signaling Cis-Elements Found in WOX promoters

Sequences correlated to hormone and environmental signaling were identified in the -1000 bp promoter regions of WUS (0) and WOX1-14 using PLACE (Higo *et al.*, 1999) & ATHENA (O'Connor *et al*., 2005). P-values from ATHENA (O'Connor *et al*., 2005). Right column shows the *WOX* members whose promoters contain the element and the number of cis elements contained is shown in the brackets.

Sequence	**p-value**	**Cis-element**	**Contained in WOX** (Repeats)
ACGTGKC	**0.8751**	**ABA/ ABRE**	**13** (1)
TGTCTC	**0.3006**	**Auxin/ ARF** binding	**0** (1), **4** (1), **5** (1), **8** (1)
TGACG		**Auxin**, **SA/ ASF-1** binding	**2** (2), **3** (1), **6** (1), **8** (1), **9** (1)
ACTTTA		**Auxin** induction	**1** (4), **2** (1), **3** (3), **4** (3), **6** (1), **7** (2), **8** (1), **9** (1), **10** (1), **14** (2)
CATATG		**Auxin** response	**1** (2), **3** (3), **5** (1), **10** (1)
GGWAGGGT		**Ammonium** response	**3** (1)
AAACAAA		**Anaerobic fermentative**	**0** (3), **1** (1), **2** (2), **3** (1), **4** (1), **5** (3), **6** (4), **7** (1), **8** (1), **9** (1), **11** (1), **12** (1), **13** (1), **14** (2)
TCATCAC			**1** (1), **11** (2)
NGATT		**Cytokinin/ ARR1** binding	**0** (9), **1** (11), **2** (8), **3** (9), **4** (9), **5** (12), **6** (17), **7** (18), **8** (15), **9** (11), **10** (6), **11** (10), **12** (14), **13** (12), **14** (14)
AWTTCAAAA		**Ethylene** response	**2** (1), **4** (1), **5** (1), **9** (1), **10** (1), **14** (1)
GCCGCC	**0.1862**		**2** (1), **13** (1)
CCTTTT		**GA** response/ **BPBF** binding	**0** (1), **1** (2), **3** (3), **6** (3), **8** (2), **9** (1), **10** (1), **11** (1), **12** (4), **14** (1)
TAACAAA		**GA/MYB** binding	**3** (1), **4** (2), **6** (1), **9** (1), **10** (1), **12** (1), **14** (2)
TGAC		**GA/WRKY** binding	**0** (5), **1** (4), **2** (8), **3** (3), **4** (5), **5** (5), **6** (5), **7** (5), **8** (7), **9** (9), **10** (7), **11** (2), **12** (5), **13** (10), **14** (8)
ACGTGTC	**0.6995**	**GA** down regulated	**13** (1)
T_6CC		**GA** induction	**2** (4), **6** (1), **7** (1), **13** (1)
MACCWAMC	**0.9154**	**GA /MYB** binding	**1** (1), **9** (1)
TAACAAR	**0.4252**	**GA** response	**1** (1), **3** (1), **4** (1), **6** (1), **9** (1), **10** (1), **12** (1), **14** (1)
AACGTG		**JA/ JAMYC2, 10** binding	**1** (1), **8** (1)
GRWAAW		**Light** response	**0** (16), **1** (8), **2** (21), **3** (6), **4** (14), **5** (5), **6** (13), **7** (15), **8** (24), **9** (8), **10** (9), **11** (12), **12** (10), **13** (15), **14** (13)
GATAA	**0.7755**	**Light/I-box**	**1** (7), **2** (3), **3** (2), **4** (2), **5** (2), **6** (4), **7** (6), **8** (5), **9** (2), **10** (2), **11** (4), **12** (1), **13** (2), **14** (1)
YTCANTYY			**0** (5), **1** (3), **2** (2), **3** (3), **4** (5), **5** (2), **6**

			(2), **7** (1), **8** (2), **10** (2), **11** (2), **13** (1), **14** (3)
ATACGTGT	**0.2883**	**Light/Z-box**	**12** (1)
GANTTNC		**LCR1** binding	**0** (2), **1** (2), **2** (1), **3** (1), **6** (3), **8** (3), **9** (2), **10** (1), **12** (2), **13** (1), **14** (3)
CANNTG		**MYB/ BHLH** binding	**0** (2), **1** (5), **2** (1), **3** (4), **4** (5), **5** (4), **6** (3), **7** (2), **8** (1), **9** (8), **10** (10), **11** (8), **12** (10), **13** (4), **14** (6)
GTTAGTT	**0.0142**	**MYB/ERF**	**2** (1), **7** (1), **9** (1), **11** (1), **12** (2), **13** (1)
MTCCWACC	**0.5206**	**MYB1** binding	**1** (1), **8** (1), **9** (1), **12** (1), **13** (1)
TAACTSGTT	**0.1865**	**MYB2** binding	**7** (1)
TAACTG	**0.2683**		**0** (1), **7** (2), **8** (1), **13** (2), **14** (1)
TAACTAAC	**0.5022**	**MYB3** binding	**7** (1)
AMCWAMC	**0.3228**	**MYB4** binding	**0** (1)**, 1** (2)**, 2** (2)**, 6** (1)**, 7** (2)**, 8** (1)**, 9** (1)**, 11** (1)**, 12** (2)**, 13** (2)**, 14** (3)
CTAACCA	**0.4496**	**MYB** recognition	**3** (1), **14** (1)
CNGTTR			**0** (1), **1** (1), **3** (1), **4** (1), **6** (1), **7** (4), **8** (1), **9** (2), **10** (3), **11** (3), **12** (3), **13** (8), **14** (2)
WAACCA	**0.2044**		**1** (2), **2** (1), **3** (3), **4** (2), **6** (2), **7** (1), **8** (2), **9** (2), **10** (1), **11** (2), **12** (2), **13** (3), **14** (5)
CATGTG	**0.6882**	**MYC** recognition	**4** (1)**, 5** (1), **6** (2), **8** (1)
CACATG	**0.6882**	**MYC2** binding	**4** (1)**, 5** (1), **6** (2), **8** (1)
AAMAATCT	**0.1178**	**Phytochrome/CCA1** binding	**1** (1), **5** (1), **6** (2), **7** (1), **13** (1)
AACCAA		**Phytochrome** regulation	**1** (1), **3** (2), **4** (3), **6** (2), **8** (2), **10** (1), 11 (1), **13** (4), **14** (2)
GCCAC		**PHYA** induced	**2** (1), **4** (1), **5** (1), **6** (1), **9** (1), **12** (1)
GAGTGAG			**9** (1)
ATAAAACGT			**8** (1), **9** (1)
GGGCC			**3** (1), **5** (1)
TGTATATAT			**3** (1), **4** (1), **5** (2), **6** (1), **7** (1), **12** (2)
TTGAC	**0.5481**	**SA/ WRKY** binding	**2** (3), **4** (4), **5** (1)
AATAGAAAA		**Sucrose** regulation	**1** (1), **2** (1), **7** (1), **14** (1)
TACGTA		**Sugar** respression/**RITA1** binding	**3** (1), **5** (1), **7** (1), **8** (1)
TTATCC		**Sugar** response	**2** (1), **5** (1), **7** (2)
GCGGCAAA		**UV-C** response	**2** (2)

Table 1.C.1 Somatic Embryogenesis Response to Light and Darkness

The effect of light on somatic embryogenesis of *Arabidopsis thaliana* Columbia wild type and wox7 mutant. Immature, zygotic bent-cotyledon explants were under induction for 14 days followed by 7 days of maturation. Induction light condition is listed on the left of -> and maturation light condition is listed on the right. The percentage of somatic embryo forming callus (%SEFC ± SE), number of somatic embryos per SEFC (SE/SEFC ± SE) and total somatic embryos per explants (Total SE ± SE). Four replicates of 30 explants were used per line. SE = standard error.

	%SEFC± SE	SE/SEFC± SE	Total± SE
WT Light -> Light	59%±7%	2.44±0.28	43.00±5.40
wox7 Light -> Light	57%±19%	3.12±0.23	53.00±10.23
WT Light -> Dark	50%±4%	2.24±0.23	32.20±3.00
wox7 Light -> Dark	69%±20%	2.51±0.09	51.67±8.02
WT Dark -> Light	36%±19%	1.44±0.22	17.25±6.47
wox7 Dark ->Light	46%±14%	1.41±0.04	18.86±3.19
WT Dark -> Dark	28%±9%	1.06±0.04	8.85±1.48
wox7 Dark -> Dark	33%±6%	1.12±0.05	11.25±1.31

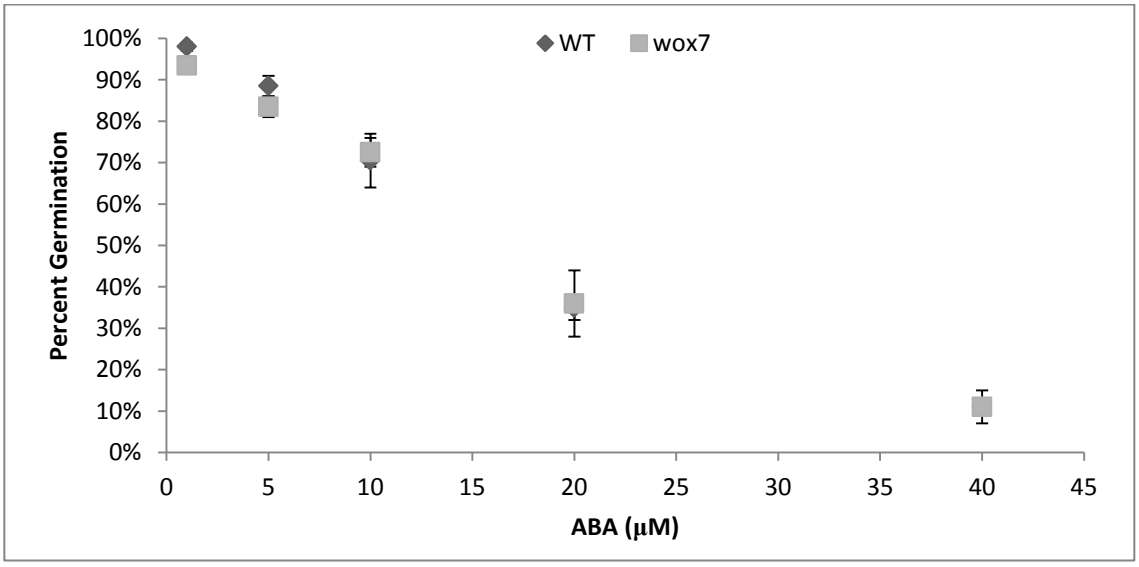

Fig 1.B.1 ABA Inhibition of Light Germination of wox7 Seeds

ABA inhibition of light germination of 6 month old seeds of Col-0 (WT) and *wox7*. Germination ± standard deviation was measured 7 days post stratification (3 days, 4°C). Three replicates of 50 seeds were sown on ½ MS media + 1% sucrose with 1, 5, 10, 20 and 40 µM ABA and kept under white light at 22±2°C. Significance of difference from WT: * $p \leq 0.05$ (One-way ANOVA). No significant difference was found.

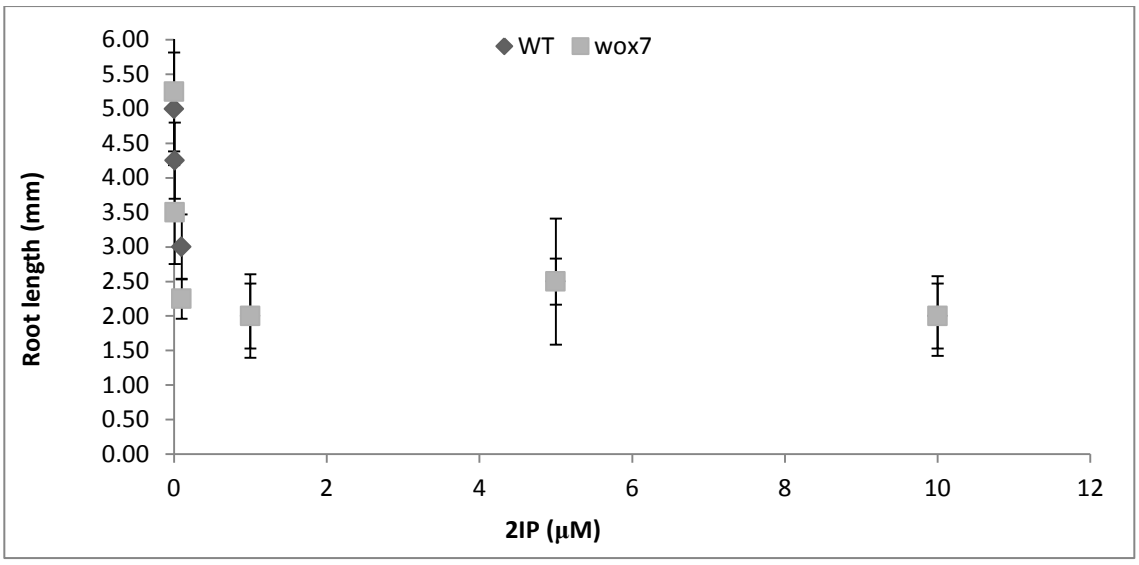

Fig 1.B.2 Dark Root Growth Inhibition of wox7 Seedlings by Cytokinin

Cytokinin inhibition of root elongation of Col-0 and *wox7* seedlings. Root length ± standard deviation was measured for 4 day old light grown seedlings after transfer to darkness for 7 days (22±2°C). Three replicates of 5 Col-0 and 5 *wox7* seedlings were sown in alternation on ½ MS plates with 2% sucrose at 0.05, 0.1, 0.5, 1, 5, 10 µM 2IP. Significance of difference from WT: * $p \leq 0.05$ (One-way ANOVA). No significant difference was found.

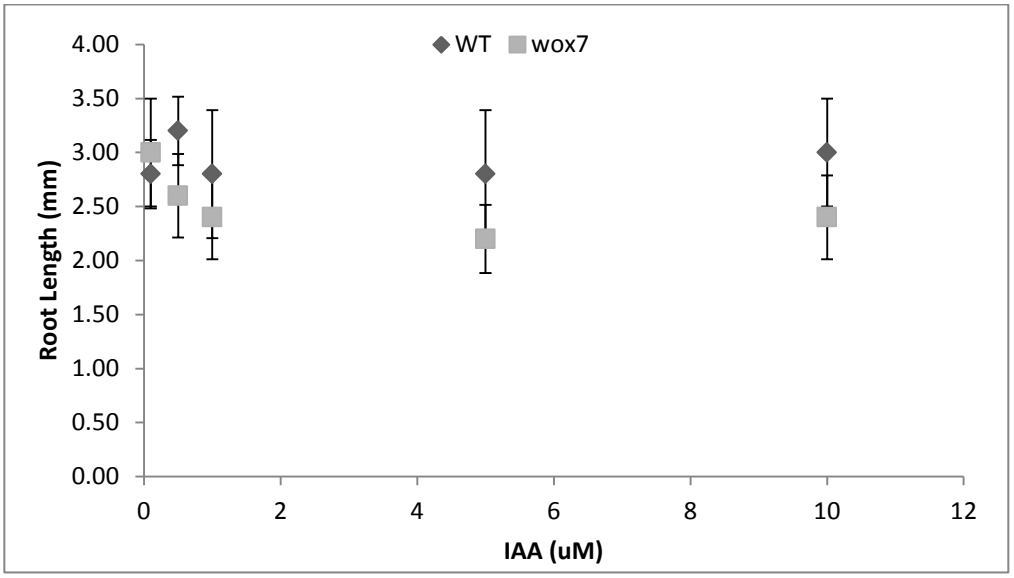

Fig 1.B.3 Dark Root Growth Inhibition of wox7 Seedlings by Auxin

Auxin inhibition of root elongation of Col-0 and *wox7* seedlings. Root length ± standard deviation was measured for 4 day old light grown seedlings after transfer to darkness for 7 days (22±2°C). Three replicates of 5 Col-0 and 5 *wox7* seedlings were sown in alternation on ½ MS plates with 2% sucrose at 0.1, 0.5, 1, 5, 10 µM IAA. Significance of difference from WT: * $p \leq 0.05$ (One-way ANOVA). No significant difference was found.

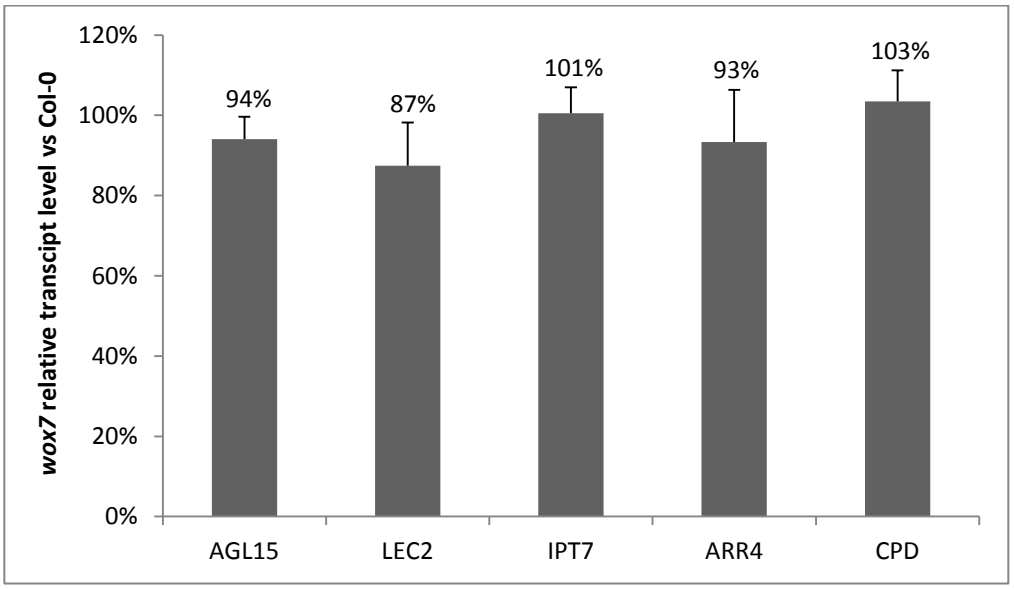

Fig 1.C.1 Relative Transcript Level of wox7 to wild type at Day 5 of SE Induction
The relative transcript levels ± standard deviation in*wox7* to Col-0 wild type at day 5 of somatic embryogenesis induction under light of the genes *AGAMOUS LIKE 15*(*AGL15),* LEAFY COTYLEDON 2 (*LEC2*), *ISOPENTENYLTRANSFERASE 7* (*IPT7*), *ARABIDOPSIS RESPONSE REGULATOR 4* (*ARR4*) and *CONSTITUITIVE PHOTOMORPHOGENIC DWARF* (*CPD*) were measured using real time PCR. Three replicates of 50 pooled embryos were used. Significance of difference from WT: * $p \leq 0.05$ (One-way ANOVA). No significant differences were found.

Table 2.A.1 Somatic Embryogenesis Response to Light Quality
The effect of light quality during somatic embryogenesis induction of *Arabidopsis thaliana* Col-0 relative to continuous white light induction. All explants were matured in complete darkness. Far Red 740 nm; Red 660 nm; Green 540 nm; Blue 470 nm. Percentage of callus forming somatic embryos (%SEFC ± SE), somatic embryos per SE forming callus (SE/SEFC ± SE) and total embryos per explant (Total ± SE) were measured from 30 explants with 4 replicates. SE = standard error.

	%SEFC± SE	SE/SEFC± SE	Total SE± SE
Dark	34%±2%	1.30±0.06	13.58±1.64
Far Red	51%±7%	1.39±0.05	20.91±2.81
Red	49%±4%	1.78±0.07	26.46±3.27
Green	38%±5%	1.42±0.09	16.36±2.45
Blue	29%±8%	1.03±0.18	9.76±4.16
White	50%±2%	1.79±0.08	26.98±0.70

Table 2.B.1 Somatic Embryogenesis Response of phy Mutants

The somatic embryogenesis competence of Arabidopsis phytochrome mutants during somatic embryogenesis relative to the Col-0 wild type. Percentage of callus forming somatic embryos (%SEFC ± SE), somatic embryos per SE forming callus (SE/SEFC ± SE) and total embryos per explant (Total ± SE) were measured from 30 explants with 4 replicates. SE = standard error.

	%SEFC± SE	SE/SEFC± SE	Total± SE
phyA	53%±3%	2.82±0.28	46.47±7.08
phyB	53%±6%	2.82±0.26	45.33±6.72
phyD	68%±4%	2.79±0.11	57.10±3.77
phyE	55%±11%	1.92±0.13	31.86±3.81
Col WT	52%±9%	2.71±0.26	42.55±6.96

Table 2.C.1 Somatic Embryogenesis Response of agl15, bes1 and serk Mutants

The effect of *agl15*, *bes1*, *bri1* or *serk1-5* mutation during somatic embryogenesis in *Arabidopsis thaliana* relative to Columbia wildtype. Percentage of explants forming somatic embryos (%SEFC ± SE), somatic embryos per SE forming explants (SE/SEFC ± SE) and total embryos per explant (Total ± SE) were measured from 30 explants with 4 replicates. SE = standard error.

	%SEFC± SE	SE/SEFC± SE	Total± SE
WT	50%±4%	2.24±0.27	32.20±3.00
serk1	50%±24%	1.52±0.16	22.67±1.78
serk2	49%±7%	1.91±0.06	28.24±3.30
serk3	65%±11%	2.90±0.19	55.11±2.08
serk4	59%±4%	2.08±0.12	37.05±4.66
serk5	48%±12%	1.24±0.04	17.79±2.30
bes1	56%±5%	2.71±0.43	38.11±7.21
agl15	30%±10%	1.18±0.09	9.10±1.81
bri1	51%±6%	2.46±0.39	32.74±7.19

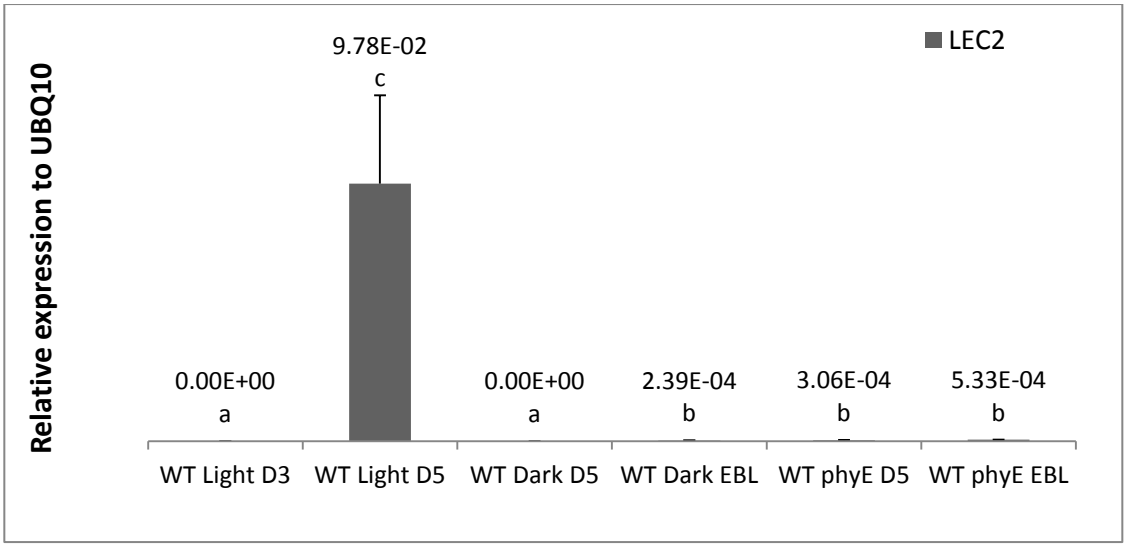

Fig 2.A.1 LEC2 Expression During SE Induction

Relative expression of *LEAFY COTYLEDON 2* (*LEC2*) versus the internal marker *UBQ10* during somatic embryogenesis induction. Explants of Col-0 background were induced under light for 3 or 5 days or under darkness for 5 days. The *phyE* mutant was induced under light. 2 µM of 24-epibrassionlide was used for EBL treatments and mRNA was taken at day 5. Three biological replicates were used for each treatment. The statistical significance between groups was $p \leq 0.001$ (One-way ANOVA, pair-wise).

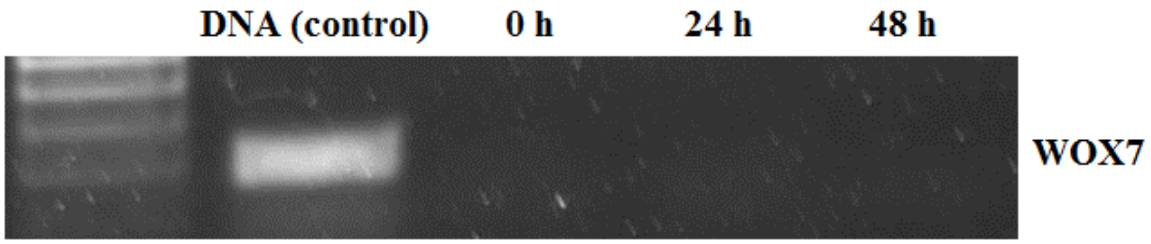

Fig 3.A.1 RT PCR of WOX7 During Dark Germination

Reverse transcriptase detection was used for full length WOX7 RNA during dark germination of Col-0. (Left to Right) genomic DNA control, 0 h, 24 h and 48 h post stratification (4^{o}C, 3 d). Pooled from three replicates of 50 pooled seeds sown on 20 µM KNO_3 plates.

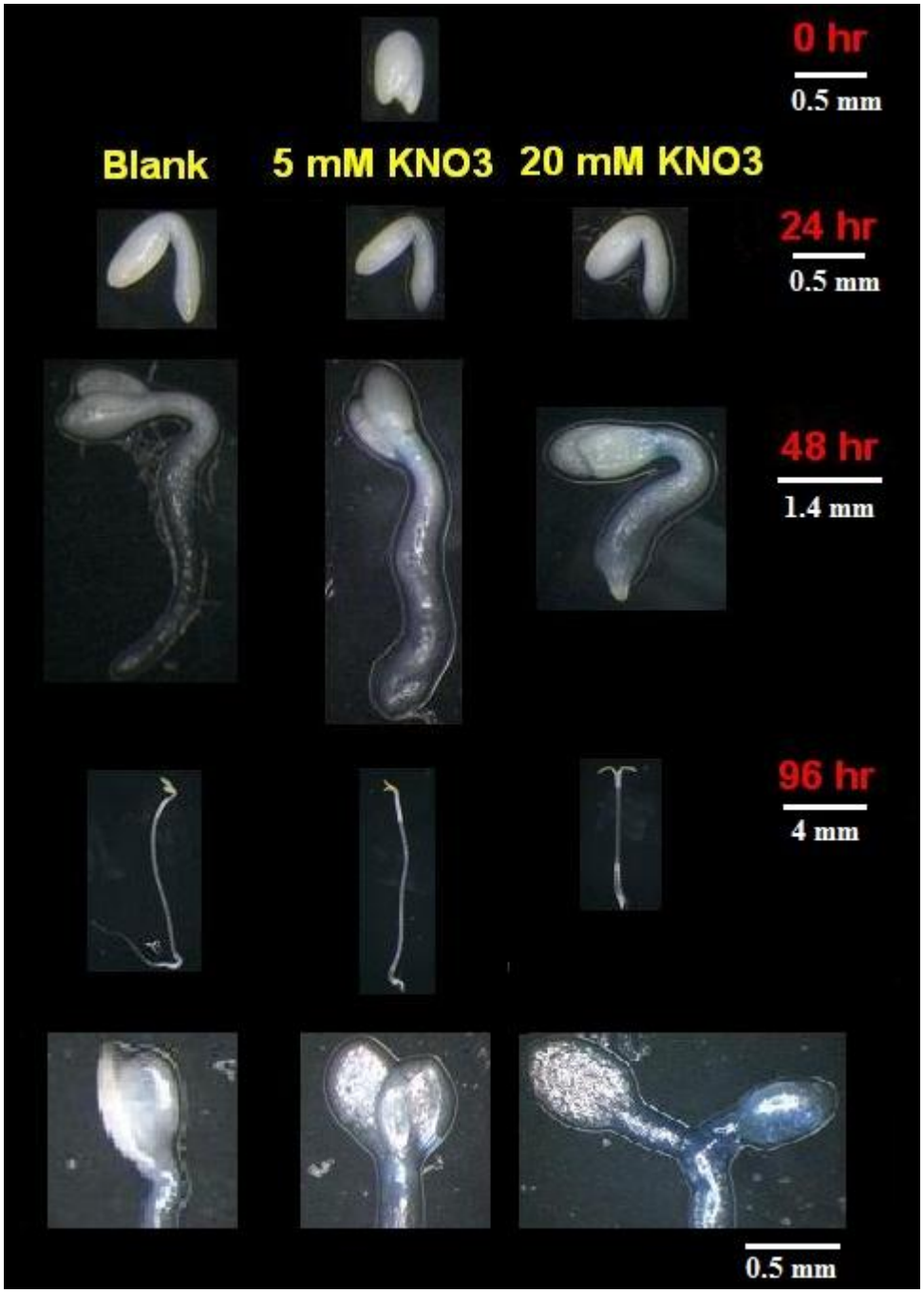

Fig 3.B.1 pWOX7::GUS Expression and Skotomorphogenesis

pWOX7::GUS expression was examined during dark germination (22±2^{o}C). Seeds were sown on agar plates with nothing (blank), 5 or 20 mM KNO_3. Plants were incubated with GUS stain for 24 hr. Representative photos were taken at 0h, 24h, 48h and 96h post stratification (4^{o}C, 3d). (0h) No GUS activity detected. (24 h) No GUS activity, no germination. (48h) GUS activity in SAM of germinated seedlings, but not in non-germinated seeds, expression is stronger at higher nitrate. (96 h) GUS activity found in shoot apex (bottom) with highest levels in open cotyledons of nitrate plates.

Table 4.A.1 Arabidopsis Mutant Lines and Confirmation Primers

Arabidopsis mutant lines were ordered from the ABRC. Confirmation primers designed using the SALK primer designer were used for SALK, SAIL and GABI-KAT lines.

Name	Gene	AGI #	ABRC stock#	Collection	Insert Site	Left Primer	Right Primer
AGAMOUS LIKE 15	AGL15	AT5G13790	SALK_076234C	SALK	exon 4	TTCGTCCTTCTATCATGTGCC	AGAACCTCCAGGAGGATTGTG
BRI1-EMS-SUPPRESSOR 1	BES1	AT1G19350	SALK_076106C	SALK	5' UTR	TCGCAACAAAATGCATATCAC	ACGGTTAAAAATGGAAATGGG
BRASSINOSTEROID INSENSITIVE 1	BRI1	AT4G39400	SALK_003371C	SALK	exon	AATACACTTGATTTTCCCGGG	TCGTTCCATTGAAGAGATTGG
GA REQUIRING 1	GA1	AT4G02780	CS3104	*ga1-3*		Verified by non-germination without GA	
NITRATE REDUCTASE 1/2	NIA1/ NIA2	AT1G77760 AT1G37130	CS6512	transposon		Verified phenotypically using NO_3 as sole nitrogen source	
PHYTOCHROME A	PHYA	AT1G09570	SALK_014575C	SALK	exon 1	CCAGTCAGCTCAGCAATTTTC	AATGCAAAACATGCTAGGGTG
PHYTOCHROME B	PHYB	AT2G18790	SALK_022035C	SALK	exon 2	CATCATCAGCATCATGTCACC	TTCACGAAGGCAAAAGAGTTG
PHYTOCHROME D	PHYD	AT4G16250	SALK_027956C	SALK	exon 1	AACCCGGTAGAATCAGAATGG	ATCGGTTACAGTGAAAATGCG
PHYTOCHROME E	PHYE	AT4G18130	SALK_092529C	SALK	exon 1	AAAGAGGCGGTCTAGTTCAGC	TATCAGTGGTTAAACCCGTCG
SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1	SERK1	AT1G71830	SALK_044330C	SALK	exon 10	ATACACAAAAGTGAAACGGCG	TAATGACACAGAGAGGCCACC
	SERK1::GUS		CS870222	SAIL	5' UTR	TTTTCAACCCAAAGAACATGC	CGGGATTACGAATCTTACTTGC
	SERK2	AT1G34210	SALK_058020C	SALK	exon 2	AGTGAAGAGCGAGAAGGAACC	AAGGCTTAGGCTTTTGTTTGG
	SERK3	AT4G33430	SALK_116202C	SALK	exon 8	CATGACATCATCATCATTCGC	ATTTTGCAGTTTTGCCAACAC
	SERK4	AT2G13790	SALK_072545C	SALK	intron 3	GTTTGTCATCAGGTGACCCTG	CAAGCTCCTCAGGTATCTCCC
	SERK5	AT2G13800	SALK_147275C	SALK	exon 8	GATGAGCTCGAGAAGCATGAC	AACATTCCACTTGGTTGATGC
WUSCHEL RELATED HOMEOBOX 1	WOX1	AT3G18010	SALK_148070	SALK	intron 3	TCTCACCATCGCTACAAAACC	GGGGTTTCTATTACATGAAAGGG
	WOX3	AT2G28610	SALK_050857	SALK	5' UTR	GATGAGAGCAGCAATCAAAGG	TGAATGATCCACCTCGAGAAC
	WOX4	AT1G46480	CS376573	GABI-KAT	exon 1	AGG CAT GCA TAG CAT TTG CT	GGA TAC ATC AAA CTC GCA GA
	WOX5	AT3G11260	SALK_084556	SALK	exon 2	TGGGTGAACGATCACTTTCTC	GCGAAGAAGATTGTCAAGAGG
	WOX6	AT2G01500	SALK_033323C	SALK	exon 2	TGGCGTATTTACCAACCTCTG	ATCACCATCAGCTCATAACCG

WUSCHEL RELATED HOMEOBOX 7	WOX7	AT5G05770	SALK_065801	SALK	exon	TACTCATTTCACGGGTAGGCC	TTCAAACCTTGGTCCATTCAC
	WOX7::GUS		CS873901	SAIL	exon	Use above with SAIL primer	
	WOX8	AT5G45980	SALK_014799	SALK	exon 2	CCCAAAATATCGTATGCATGC	CCTAAACCGGAGCAGATTAGG
	WOX10	AT1G20710	SALK_010064	SALK	5' UTR	GTAAATGGCGATTTGCTTACG	ACATGGACAGATCACGGAGAC
	WOX11	AT3G03660	SALK_004777C	SALK	intron 1	TCAAAGCAATGGACCAAGAAC	TTAGGCAATATTGGAAGCATG
	WOX12	AT5G17810	SALK_078390	SALK	5' UTR	CGAGTATCAAGATTTGCTCCG	CGCAAACGTAACTTGGAACAC
	WOX13	AT4G35550	CS875487	SAIL	exon 1	GAA AAG TAC CAA CCA ACG AC	ACC CAT ACA CCA AAG TGA AG
	LBa1.3	SALK tdna insert primer			-280	ATT TTG CCG ATT TCG GAA C	
	SAIL-L1	SAIL tdna insert primer			-314	TTA AAA ACG TCC GCA ATG TG	
	GK-L1	GABI-KAT tdna insert primer				ATATTGACCATCATACTCATTGC	

Figures 4.A PCR Confirmations of T-DNA inserts

PCR confirmation of mutant lines using (top) endogenous genomic primer pair and (bottom) insert specific primer plus complementary endogenous primer. Homozygous mutants were selected by the absence of an endogenous band and presence of the T-DNA insertion band. Not all samples (S#) tested shown, only the tests containing the confirmed homozygote mutant(s).

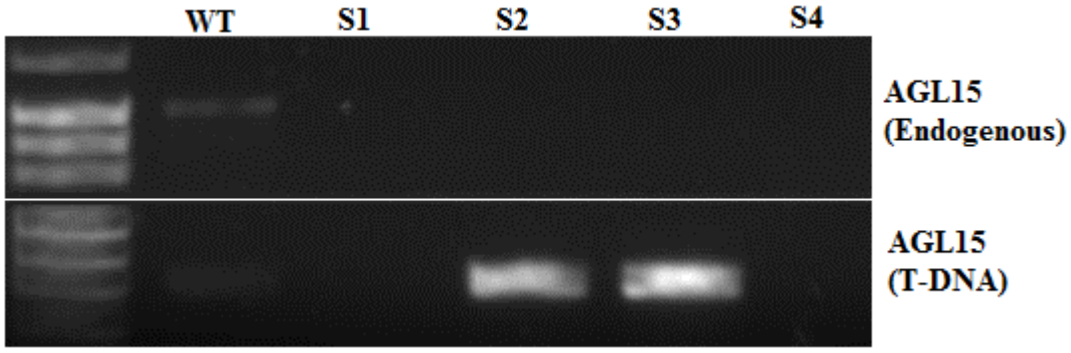

Fig 4.A.1 PCR confirmation of agl15

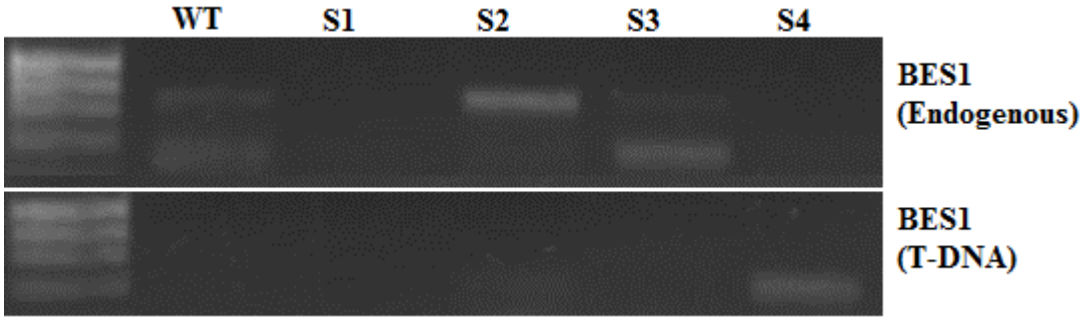

Fig 4.A.2 PCR confirmation of bes1

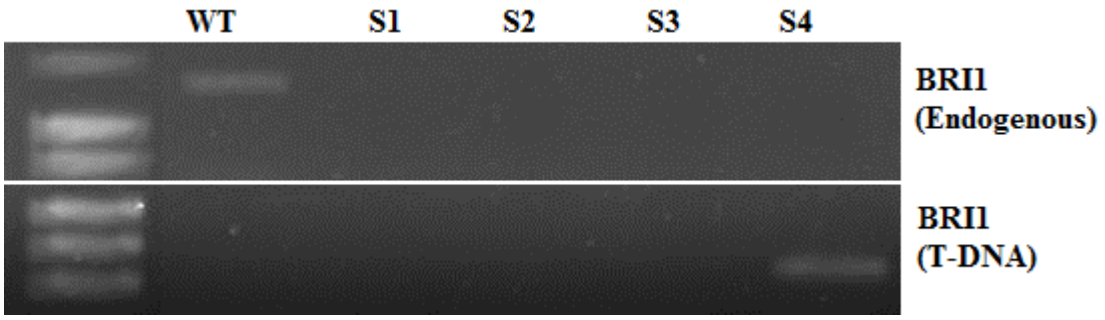

Fig 4.A.3 PCR confirmation of bri1

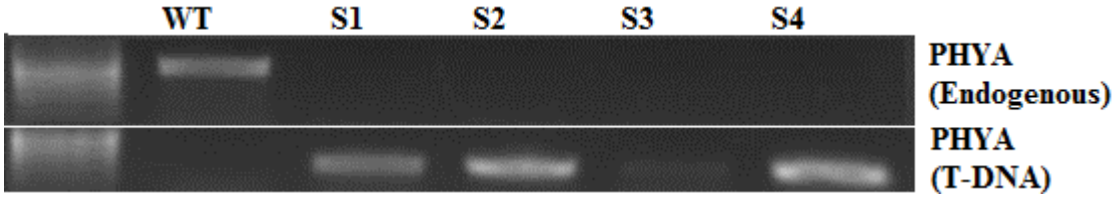

Fig 4.A.4 PCR confirmation of phyA

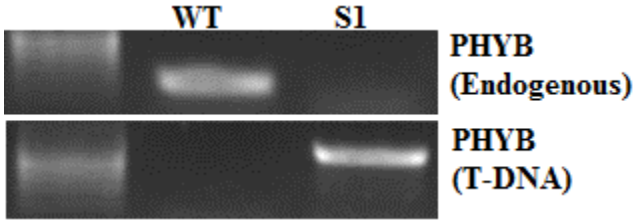

Fig 4.A.5 PCR confirmation of phyB

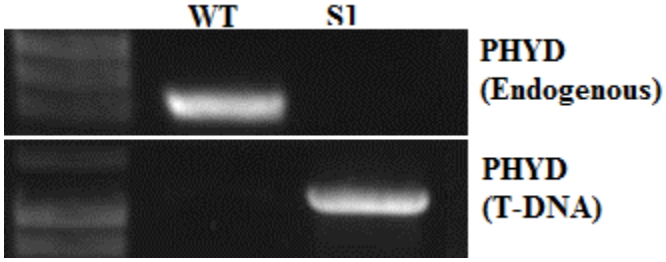

Fig 4.A.6 PCR confirmation of phyD

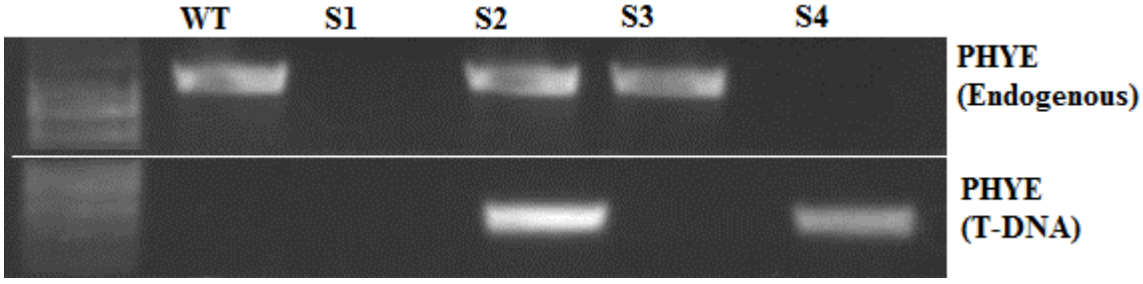

Fig 4.A.7 PCR confirmation of phyE

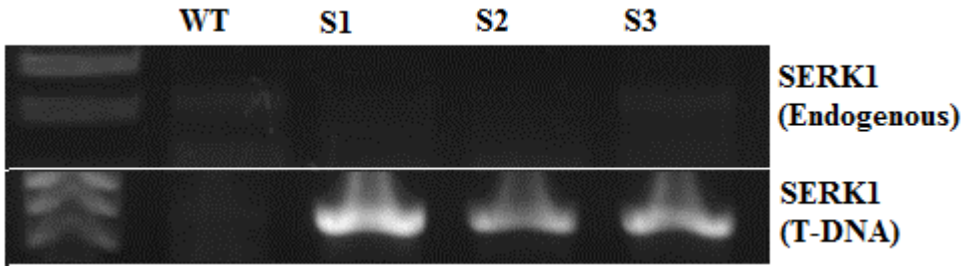

Fig 4.A.8 PCR confirmation of serk1

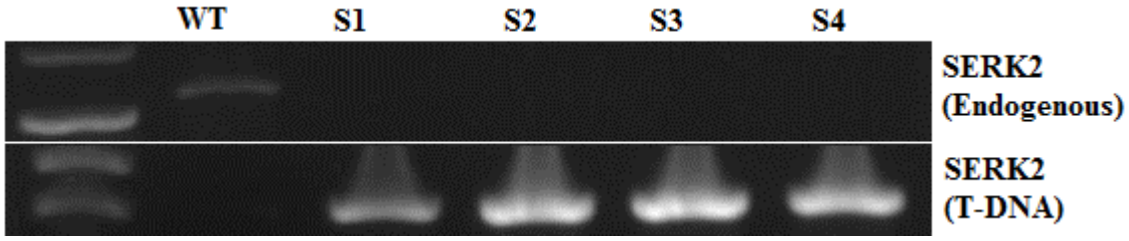

Fig 4.A.9 PCR confirmation of serk2

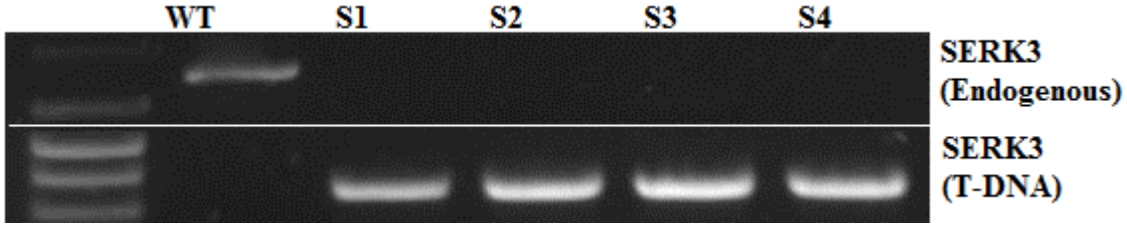

Fig 4.A.10 PCR confirmation of serk3

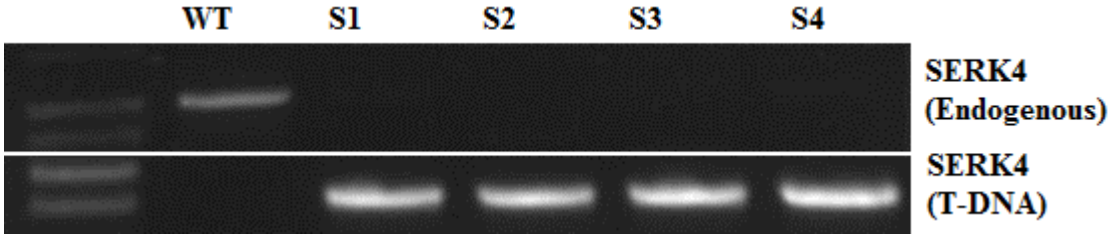

Fig 4.A.11 PCR confirmation of serk4

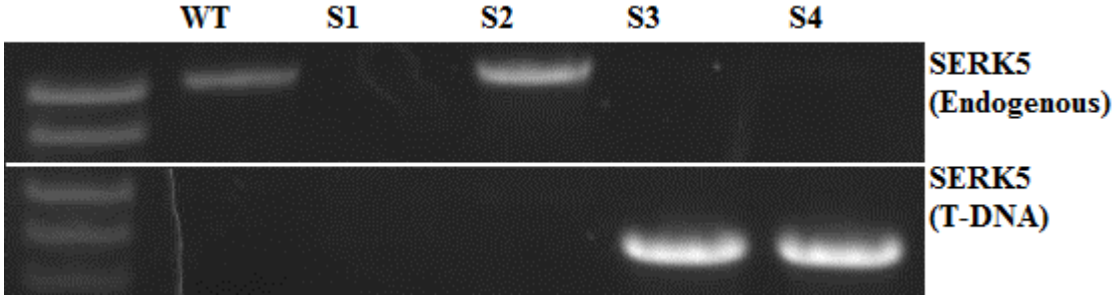

Fig 4.A.12 PCR confirmation of serk5

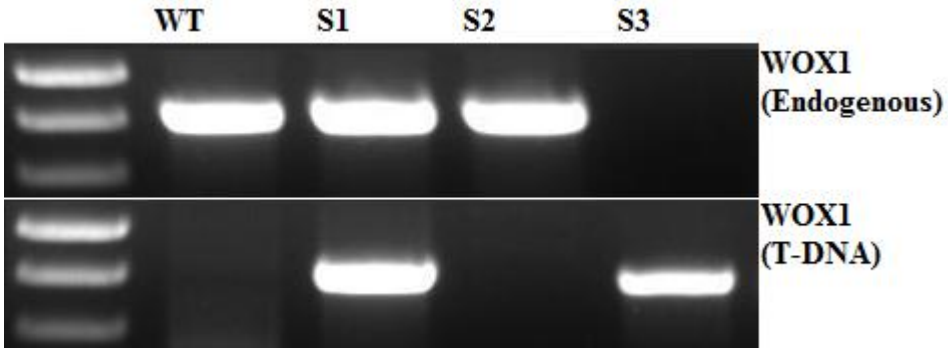

Fig 4.A.13 PCR confirmation of wox1

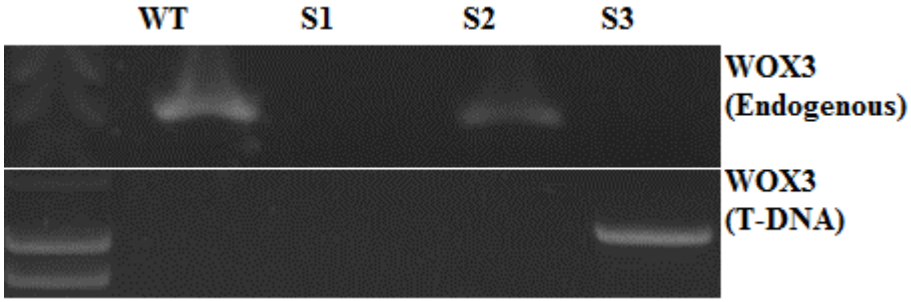

Fig 4.A.14 PCR confirmation of wox3

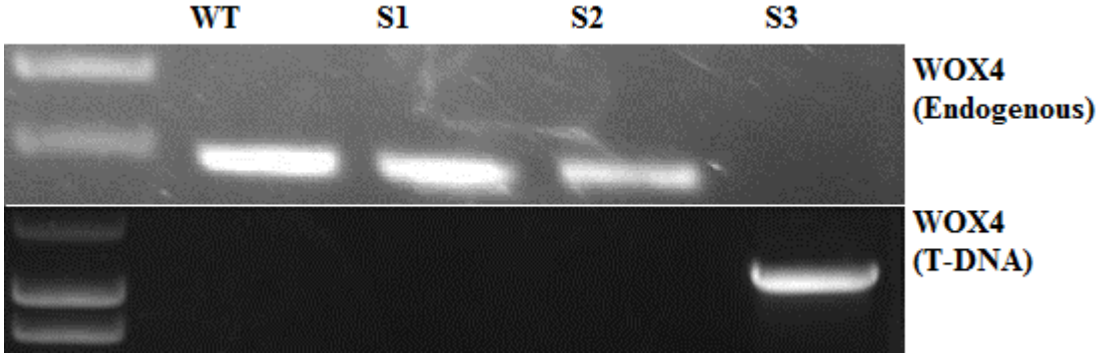

Fig 4.A.15 PCR confirmation of wox4

Fig 4.A.16 PCR confirmation of wox5

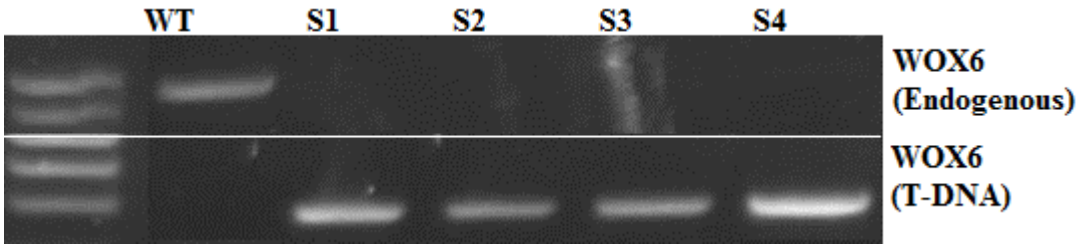

Fig 4.A.17 PCR confirmation of wox6

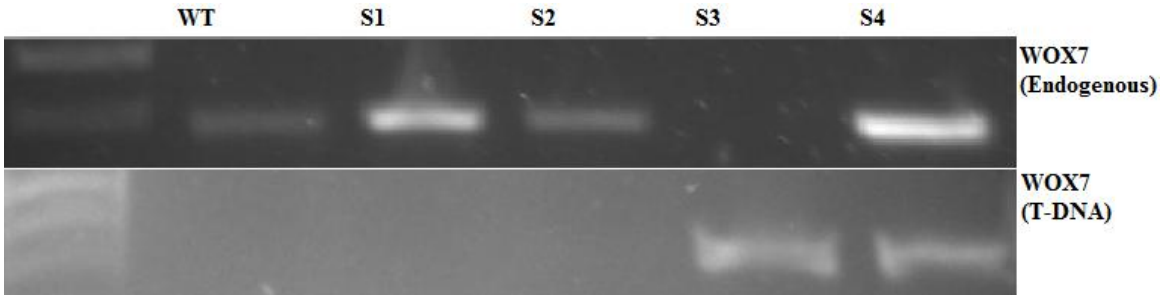

Fig 4.A.18 PCR confirmation of wox7

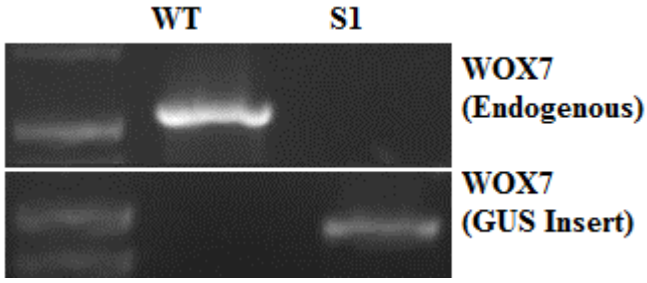

Fig 4.A.19 PCR confirmation of wox7::GUS

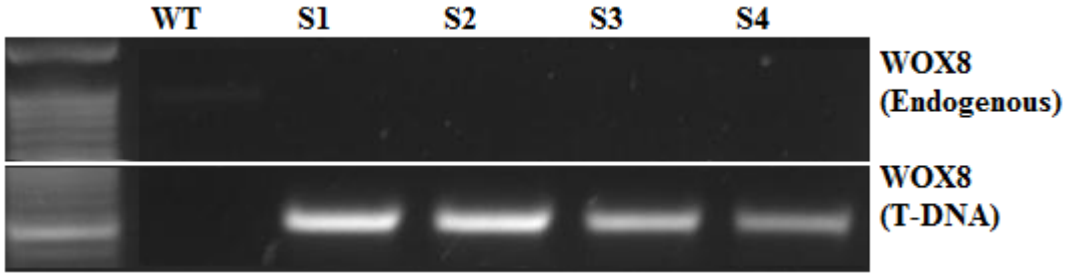

Fig 4.A.20 PCR confirmation of wox8

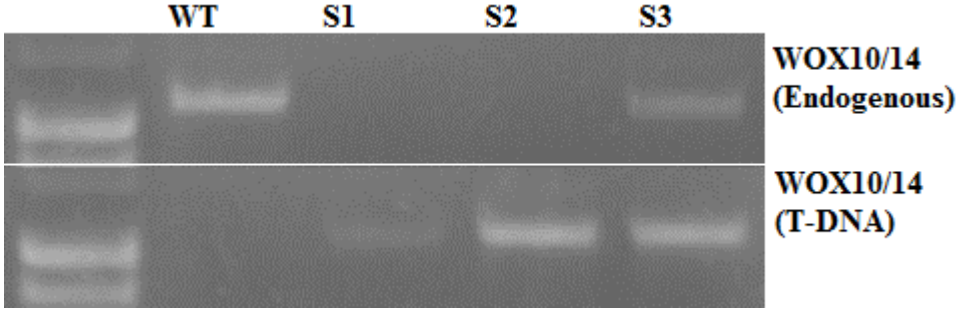

Fig 4.A.21 PCR confirmation of wox10/14

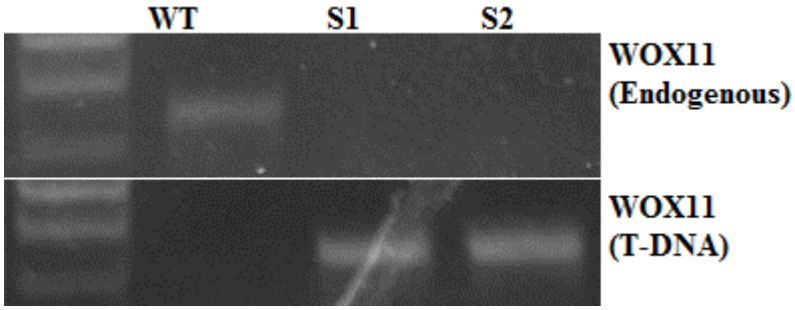

Fig 4.A.22 PCR confirmation of wox11

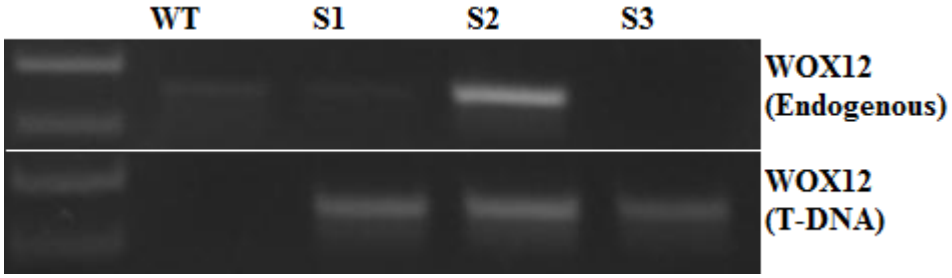

Fig 4.A.23 PCR confirmation of wox12

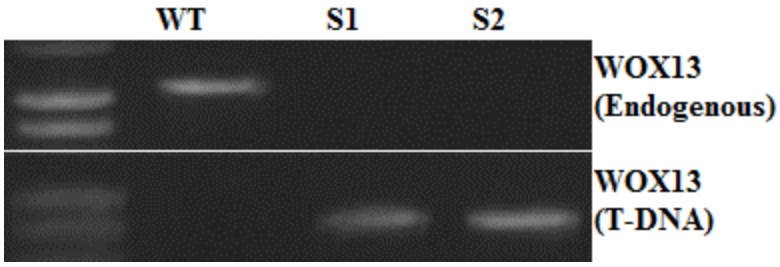

Fig 4.A.24 PCR confirmation of wox13

Table 4.A.2 Real Time PCR Primers

Real time PCR primer pairs (forward top, reverse bottom) and the primer start locations in the mRNA sequence for target genes. All primers are designed to have a T_m at 60°C. Efficiency was measured for reaction conditions listed in methods.

Gene	ATG#	Location	Primer Sequence	Efficiency
AGL15	AT5G13790	668	AACTTTGCAATTAGGGTTGCCGGG	1.20
		773	TTCGCTGCTCGTGTTTGTTGTCAC	
ARR4	AT1G10470	979	TGGAACTATTCTGGGATGCCGTGT	1.40
		1074	CGGCGTCAAGTCAACGTGCAATTA	
CPD	AT5G05690	1265	TGGCTAGGGTTGCACTCTCTGTTT	1.32
		1385	ATCGGGTACCGTTTCTGCGTTCTT	
CTS	AT4G39850	263	TGAGTGGCAACAGCTTCAAGGAGA	1.03
		416	AACCCAAGCTGTATCGTAAGCCGA	
LEC2	AT1G28300	1047	TCCTCATCAATGCCACCTGAGGAT	1.30
		1145	ACTTCCACCACCATATCACCACCA	
PHYE	AT4G18130	2907	AGCACGGACTTGAAAAGCAT	1.80
		2970	CAAGTCGAAACTCTTCTGTTTCC	
UBQ10	AT4G05320	1456	GAAGTTCAATGTTTCGTTTCATG	1.28
		1519	GGATTATACAAGGCCCCAAAA	

Table 4.B.1 Somatic Embryogenesis Induction media composition

The components of the somatic embryogenesis induction media. After dissolving all components in water, the pH is adjusted to 5.8 prior to addition of agar. Sterilization was done using by autoclaving (15 atm, 120°C, 25 min).

Reagent	*mg/L*		*mg/L*
• KNO_3	1616	• Zn-Acetate	6.2
• $CaNO_3$	408	• H_3BO_3	3
• $MgSO_4$	240	• Glycine	2
• NH_4-Citrate	189	• Nicotinic Acid	2
• $NH_4H_2PO_4$	115	• Thiamine HCl	2
• L-Glutamine	100	• Folic Acid	0.5
• Myoinosotol	100	• Pyridoxyine HCl	0.5
• Fe-Citrate	24.5	• Na_2MoO_4	0.25
• EDTA	15	• $CuSO_4$	0.025
• $MnCl_2$	12.5	• $CoCl_2$	0.025

- ***1% Sucrose***
- ***2 uM 2,4-D***
- ***Adjust to pH 5.8***