Shoot Apical Meristem Arrest in Brassica and Tomato

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Thesis

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General Introduction

Chapter 1

Introduction

The life cycle of a plant starts with the fertilised egg cell, which soon starts polar differentiation and the formation of apical meristems in the embryo. This embryo resides in a seed that germinates into a seedling which unfolds its cotyledons and starts photosynthesising to produce sugars. These sugars serve as energy for the development of leaves emerging from the apical growing tip of the plant. At a specific age of the plant this growing tip can change identity, start producing flowers that can be fertilized eventually forming fruits and new embryos. Plants unlike animals have the ability to develop plant organs after embryogenesis. These organs can be root (hairs), leaves or flowers and are formed from the plant growing tip. It is important to understand the mechanism by which plants can maintain this pluripotent state both from a biological and an applied point of view, because without a growing tip plants are not able to produce leaves or fruits, which will impair plant based food production.

The vegetative meristem and organ formation

Most of a the above ground tissues in higher plants originate from a pool of cells known as stem cells, which are located in the centre of the growing tip (Yanai, Shani *et al.* 2005) (Clark, Running *et al.* 1995). The word meristem originates from the ancient Greek “μερίζειν” (merizein), which means to divide and was first introduced by the Swiss botanist Carl Wilhelm von Nägli (Nägli 1858). This shoot apical meristem (SAM) (Yanai, Shani *et al.* 2005) can be subdivided into three different zones: the peripheral zone, the central zone and the rib zone. While the central zone acts as an reservoir of stem cells, the rib zone gives rise to the stem tissue and the peripheral zone produces the lateral organs (Steeves T.A. 1989; Meyerowitz 1997; Barton 1998). Leaves and all lateral organs of a plant are derived from three different cell layers of the meristem: the epidermal layer (L1), the sub-epidermal layer (L2) and the corpus layer (L3). This multiple derivation point of leaves from the three layers suggests a cross talk between all layers of the SAM to produce leaves (Satina and Blakeslee 1941). Besides the generation of new leaf primordia, another important

process taking place in the meristem, is the constant self-renewal to maintain a pool of pluripotent cells in the stem cell niche (Lyndon 1998). The processes and genes underlying this continuous self-renewal and production of organs are best described in the model species *Arabidopsis thaliana.* In this species an important genetic feed-back regulatory loop has been discovered that is needed to maintain and specify the stem cell niche in the SAM (Yanai, Shani *et al.* 2005). Most important players of this feedback loop are the homeobox transcription factor gene *WUSCHEL* (*WUS*) and the *CLAVATA* (*CLV*) genes (Laux, Mayer *et al.* 1996) and (Schoof, Lenhard *et al.* 2000).

Molecular regulation of meristem maintenance in *Arabidopsis*

Maintenance of the SAM is of utmost important for a plants growth. Without the SAM and its stem cells, generation of lateral organs is impossible. Plants have therefore established a robust molecular network to maintain stem cells as long as growth is required. One of the most important genes involved in this network is *WUS*, encoding a homeobox transcription factor. *WUS* is important for maintenance of pluripotency in the stem cell niche. Knock-out plants of *WUS* terminate their growth prematurely due to the consumption of the stem cell pool by developing organs, while overexpression of this gene leads to enlarged meristems and somatic embryo formation in root tissue (Laux, Mayer *et al.* 1996; Schoof, Lenhard *et al.* 2000; Zuo, Niu *et al.* 2002). *WUS* expression is first detectable in the embryo after the eight cell stage when the cells divide periclinally (Mayer, Schoof *et al.* 1998). In the full grown embryo the SAM region consists of 16 cells and in a few cells located basal to the stem cells *WUS* is expressed. The restricted expression region of *WUS* is regulated in the central zone of the SAM where, a non-cell autonomous negative-feedback loop between *CLV* genes and *WUS* lead to the maintenance of the SAM (Laux, Mayer *et al.* 1996)(Schoof, Lenhard *et al.* 2000). For this feedback-loop three CLV proteins are known to be important: CLV1 a receptor kinase, from which the expression overlaps and surrounds the *WUS* expression domain, CLV2 a receptor-like protein and CLV3 a mobile signal peptide that binds to the CLV1-CLV2 receptor complex (Clark, Running *et al.* 1993; Clark, Running *et al.* 1995; Jeong, Trotochaud *et al.* 1999). *CLV3* is proposed to work as a signal that

sharpens the boundary between stem cells and organizing centre. In addition to CLV1 and 2, the receptor kinase CORYNE (CRN) together with CLV2 can also perceive the CLV3 signal peptide (Müller, Bleckmann *et al.* 2008). The expression patterns of *WUS, CRN* and *CLV2* genes overlap in the L3 layer of the meristem, while *CLV1* expression is restricted to the CZ supporting the current model (Müller, Bleckmann *et al.* 2008; Bleckmann, Weidtkamp-Peters *et al.* 2010).The working model for this feedback loop, so far, is that the expression of the *CLV3* gene is promoted by WUS and then the CLV3 peptide is secreted to the CZ (Schoof, Lenhard *et al.* 2000) where it is bound by CLV1-CLV2 heterodimers or CLV2 / CORYNE heterotetramers, activating intracellular signalling and restricting the diffusion of CLV3 into the organizing centre (Jeong, Trotochaud *et al.* 1999). The CLV signalling restricts *WUS* expression inside the OC and suppresses *WUS* expression outside this region (Figure 1) (Jeong, Trotochaud *et al.* 1999; Reddy and Meyerowitz 2005; Bleckmann, Weidtkamp-Peters *et al.* 2010). At the same time cells with *WUS* expression stimulate the expression of *CLV* genes, forming this earlier mentioned feedback-loop to restrict *WUS* expression. In this way a balance is created between the cell proliferation activity, stimulated by *WUS* and the inhibition of cell division by the CLV proteins, which is essential for maintenance of stem cell activity but at the same time restricting growth. Supporting this model, Arabidopsis *clv3* or *clv1* mutants exhibit enlarged meristems as they fail to restrict *WUS* inside the OC and as a result *WUS* is expressed in more cells than normal, resulting in stem cell expansion and a bigger SAM (Clark, Running *et al.* 1995; Brand, Fletcher *et al.* 2000; Yadav, Tavakkoli *et al.* 2010). Likewise, overexpression of these *CLV* genes leads to early termination of the SAM (Brand, Fletcher *et al.* 2000) due to elimination of *WUS* expression. Apart from the CLV pathway, recent studies suggest more protein complexes present in the plant that can signal CLV3. One of those suggested proteins, is *LRR- RECEPTOR-LIKE PROTEIN KINASE 2* (*RPK2*). Homodimers of *RPK2* and *CLV2-CRN* heterodimers are supposed to work in parallel together with *CLV1* homodimers in regulating SAM maintenance (Kinoshita, Betsuyaku *et al.* 2010). The regulatory feedback-loop controlling meristem maintenance of *WUS* via the *CLV* signalling pathway is well understood. In contrast, which pathways are regulated by *WUS* is far less unravelled. Nevertheless, a recent target gene analysis shed first light on genes and processes that probably are under direct control of this key meristem regulating gene (Busch, Miotk *et al.* 2010)

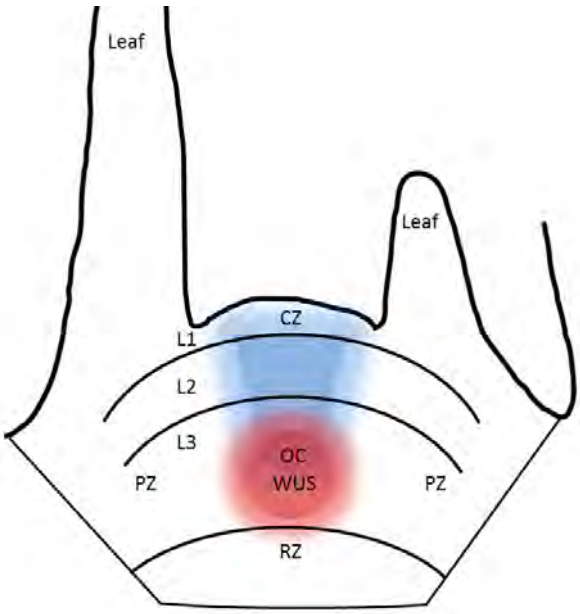

Figure 1:
Meristem structure. The shoot apical meristem of most plants can be subdivided into three layers (L1, L2 and L3) and also into zones that separate fast dividing cells in the peripheral zone (PZ) from slowly dividing cells in the central zone (Benková, Michniewicz et al. 2003). There is a third zone, the rib zone (RZ) that gives rise to the cells in the stem. The stem cells (blue) are located throughout all three layers above the organizing centre (OC) (Jeong, Trotochaud et al. 1999), where the transcription factor gene WUS is expressed.

Hormonal control of meristem maintenance

Next to the robust molecular network, plant hormones also play an important role in meristem maintenance. From tissue culture experiments it is known that elevated cytokinin signalling promotes high expression levels of *WUS.* This increased *WUS* expression changes the fate of the *WUS* surrounding cells to stem cells (Gordon, Heisler *et al.* 2007). An additional link between *WUS* and cytokinin was established when it was demonstrated that WUS represses genes from the type-A *ARABIDOPSIS RESPONSE REGULATOR (ARR)* gene family that are induced by cytokinin and themselves negatively regulate cytokinin signalling (D'Agostino, Deruère *et al.* 2000; To, Haberer *et al.* 2004; Leibfried, To *et al.* 2005). This means that *WUS* promotes cytokinin signalling. Furthermore, plants treated with cytokinin have an increased *WUS* but decreased *CLV* expression, leading to a *CLV* loss-of-function phenotype (Lindsay, Sawhney *et al.* 2006), (Clark, Running *et al.* 1993). This connects not only *WUS* but also the *WUS/CLV* feedback-loop to the downstream signalling network of cytokinin. However, it has been shown that the cytokinin induced *WUS* expression can occur independently from the reduction in *CLV* signalling, indicating that the

effect of cytokinin on *WUS* could be direct (Lindsay, Sawhney *et al.* 2006). The role of cytokinin in SAM maintenance is supported by the finding that mutants producing less cytokinins have smaller and less active SAMs (Werner, Motyka *et al.* 2003). Furthermore, evidence is obtained by computational modelling and experimental data that reveal a control of stem cell niche size and positioning via multiple feedback loops between WUS/CLV and cytokinin signalling (Gordon, Chickarmane *et al.* 2009; Chickarmane, Gordon *et al.* 2012). Besides its effect on WUS*,* cytokinin also affects *SHOOT MERISTEMLESS (STM)* expression, a Class-*I KNOTTED*-like homeobox transcription factor, which is expressed throughout the non-organogenic regions of the SAM, but is excluded from regions where primordia formation takes place (Long, Moan *et al.* 1996; Rupp, Frank *et al.* 1999; Scofield, Dewitte *et al.* 2007). *STM* has a role in preventing stem cell differentiation, which is complementary to the function of *WUS* in specifying a subset of cells as stem cells (Lenhard, Jürgens *et al.* 2002) (Williams and Fletcher 2005). The specific role of *STM* becomes clear when it is mutated or overexpressed leading either to embryos that fail to develop a meristem (Long, Moan *et al.* 1996), or to plants that are inhibited in differentiation (Gallois, Woodward *et al.* 2002). The transcription factor STM itself promotes cytokinin synthesis through the activation of *ATIPTs,* a key gene in the cytokinin synthesis pathway (Kakimoto 2001) (Jasinski, Piazza *et al.* 2005) (Yanai, Shani *et al.* 2005). In line with this link, *ATIPT*s mutations cause a decrease in meristem size, endorsing the positive role of cytokinin on the control SAM activity (Miyawaki, Tarkowski *et al.* 2006). Additionally to the promoting role of *STM* on cytokinin biosynthesis, *KNOX* transcription factors play a role in the repression of the biosynthesis of yet another phytohormone called gibberellin (GA), which is known to be a growth regulator. In addition to this repressive activity of the *KNOX* genes, cytokinins reinforce the low GA-levels by stimulating the expression of genes involved in GA catabolism (Jasinski, Piazza *et al.* 2005) (Wolters and Jürgens 2009). Thereby, *KNOX* genes are maintaining division and preventing differentiation through activation of cytokinin and repression of GA synthesis (Wolters and Jürgens 2009; Zhao, Andersen *et al.* 2010). This balance is also needed for meristem maintenance (Shani, Yanai *et al.* 2006). In rice, an important link between cytokinin and meristem maintenance was found with the discovery of an enzyme with phosphoribohydrolase activity. This enzyme is encoded by the *LONELY GUY* (*LOG*) gene, which name is based on

its single stamen mutant phenotype in the flowers. It converts inactive forms of cytokinin into active ones (Kurakawa, Ueda *et al.* 2007). In Arabidopsis, nine *LOG* genes are predicted to exist based on sequence similarities. Out of these nine, *LOG7* seems to be the most important gene for SAM maintenance. It is expressed in the SAM and supposed to play a role in cytokinin activation (Tokunaga, Kojima *et al.* 2012).

Besides cytokinin and gibberellin, a third plant hormone auxin is known to be involved in a plethora of developmental processes (Vanneste and Friml 2012). It has been shown that the precise deposition of auxin to the SAM surface can initiate organ formation and determines primordia positioning (Reinhardt, Mandel *et al.* 2000). Furthermore, auxin plays a critical role in meristem maintenance. Studies suggest that auxin deficiency induced by the inhibition of auxin transport through the chemical NPA, leads to a reduction in *WUS* and an increase in *CLV3* expression levels (Zhao, Andersen *et al.* 2010). The most abundant form of auxin is indole-3-acetic acid (IAA) and its production is controlled by the *YUCCA* genes (Bartel 1997) (Zhao, Christensen *et al.* 2001). Auxin maxima in the central zone are established via production and transport throughout the tissues. This transport is mediated by auxin importers and exporters and therefore auxin signalling or transport mutants can exhibit similar phenotypes as auxin production mutants (Zhao, Andersen *et al.* 2010). Additionally to the fact that *yucca* mutants are partially deficient in auxin, the expression levels of the genes *ARR7* and *ARR15* are increased (Cheng, Dai *et al.* 2007). These two genes are known to repress cytokinin response (To, Haberer *et al.* 2004). Therefore, auxin supresses the expression of *ARR7* and *ARR15* genes in the CZ and PZ via a gene called *MONOPTEROS* (MP) (Bleckmann, Weidtkamp-Peters *et al.* 2010) . When *MP* expression is absent, plants overexpress the two *ARR*s in exactly these zones of the SAM (Zhao, Andersen *et al.* 2010). This links the suppression of cytokining signalling via *ARR7* and *ARR15* to the *MP*-mediated auxin signalling.

All these previously mentioned regulatory interactions demonstrate a synergistic role between the plant hormones auxin, cytokinins and gibberellins in the maintenance and differentiation of the SAM.

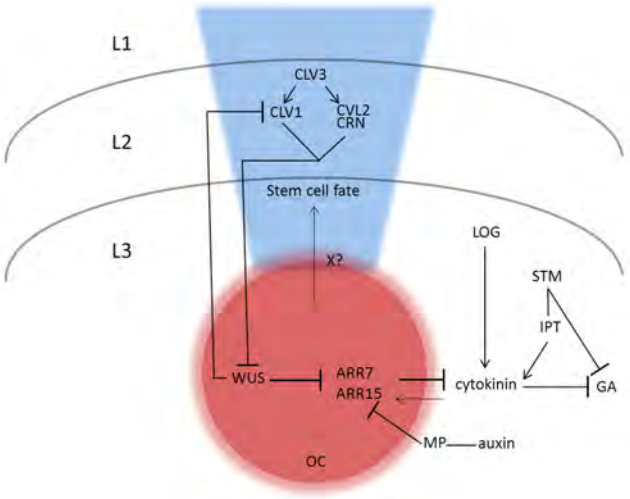

Figure 2:
Partial network of SAM regulation. The stem cell fate (blue) is maintained via an unknown signal that is generated by the *WUS*-expressing cells in the organising centre (OC, red). The signal glycopeptide CLV3, which is expressed in the stem cells, is perceived by the CLV1- and CRN-/CLV2 receptor complexes. The KNOX protein STM stimulates cytokinin biosynthesis and inhibits gibberellins. WUS itself prevents cytokinin suppression by repressing ARR7 and ARR15.

Transcriptional and hormonal regulation of organ initiation

For growth, plants need to produce cells that differentiate into tissues. While it is very important for the stem cell niche to maintain its pluripotency, the SAM is also the growing point of a plant from where all aerial plant organs are initiated and formed and therefore parts of the cells need to differentiate. Two antagonistic pathways control cell differentiation in the PZ, where leaf primordia develop from cells derived from the SAM (Yamaguchi, Nukazuka *et al.* 2012). As soon as cells are recruited into an organ, genes that are normally necessary for meristem identity specification, need to be switched off. The MYB transcription factor *ASYMMETRIC LEAVES* 1 (*AS1*) keeps these meristem identity genes repressed, specifically in organ primordia (Byrne, Barley *et al.* 2000). For the recruitment of founder cells, transcription factors involved in auxin signalling, like MP, are needed. In addition to the role of auxin in meristem maintenance a spatial auxin transport and signal is also needed for the formation of organs (Reinhardt, Pesce *et al.* 2003)(Aida, Vernoux *et al.* 2002). Besides auxin, several transcription factors have been identified as being important for the formation or specification of organ primordia. The Arabidopsis organ primordia boundary domain is defined by members of the *NAC* family of transcription factors called *CUP-SHAPED COTELYDON* 1, 2 and 3 (*CUC1*, *CUC2*, *CUC3*)

(Figure 3). *HANABA TARANU* (*HAN*) encodes another transcription factor whose expression defines the boundary between the central domain of the meristem and the organ primordia region. Additionally, *HAN* controls the number and position of *WUS*-expressing cells, independently of the CLV pathway (Zhao, Medrano *et al.* 2004; Miwa, Kinoshita *et al.* 2009).

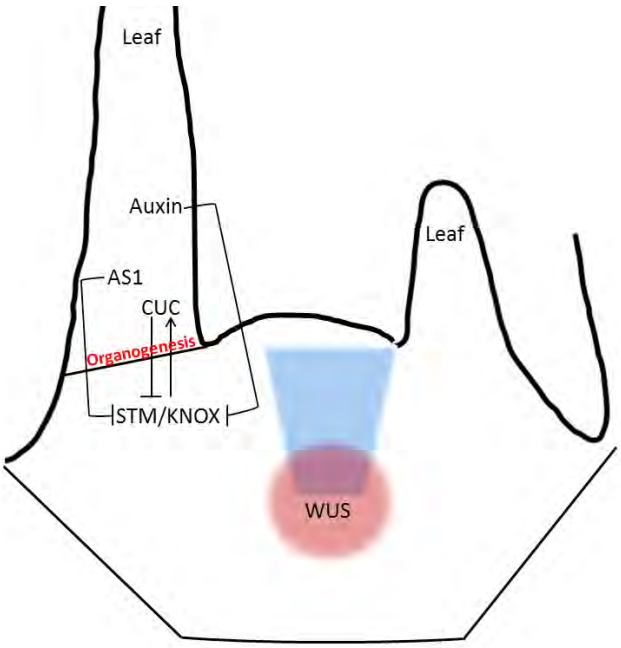

Figure 3:
Organogenesis. In cells that are recruited to form an organ, genes (e.g *STM*, KNOX) that are normally necessary for meristem identity specification need to be switched off. The MYB transcription factor *ASYMMETRIC LEAVES* 1 (*AS1*) keeps the meristem identity genes repressed in organs. Also the plant hormone auxin represses these genes in the organs. Furthermore the organ primordia boundary domain is defined by members of the NAC family of transcription factors called *CUP-SHAPED COTELYDON (CUC).*

Losing the balance, genes important for maintaining the meristem

The intrinsic network of genes regulating meristem maintenance can be disturbed by the loss of several genes. In our study we are interested in which genes can disturb the balance between meristem maintenance and differentiation, because these genes could ultimately be responsible or have an effect on genes causing meristem arrest. Therefore, we were interested in genes leading to the differentiation of the SAM and genes that are involved in meristem maintenance and not only in the formation of the meristem in the embryo.

Most of the genes involved in meristem maintenance change the expression of one of the components of the *CLV*/*WUS* pathway. *OBERON1* (*OBE1*) and *OBERON2* (*OBE2*) are plant homeodomain finger proteins. Most of *obe1* and *obe2* double mutants form only the first leaf pair and eventually die after 10-30 days of development. In these double mutants the expression of *CLV3* and *WUS* is lower during embryo development and absent after the heart stage of the embryo, indicating that *OBE1* and *OBE2* function in the regulation of the *CLV*-*WUS* pathway (Saiga, Furumizu *et al.* 2008). However, it is most likely that these two proteins are required for proper SAM establishment through meristem maintenance genes rather than for meristem specification itself (Saiga, Furumizu *et al.* 2008)(Miwa, Kinoshita *et al.* 2009). Mutant plants of *SPLAYED* (*SYD*) a SNF2 chromatin-remodelling ATPase undergo premature termination of the SAM. This termination occurs due to the loss of a direct interaction of *SYD* with *WUS* in the organizing centre, leading to strong reduction in expression of *WUS* (Kwon, Chen *et al.* 2005). *STYMPY (STIP)* a homeobox gene also known as *WOX9,* promotes growth in the vegetative meristem. In *stip* mutant plants the SAM differentiates six days after germination and these mutants lack both *CLV3* and *WUS* expression, indicating that *STIP* is needed for the maintenance of *CLV3* and *WUS* expression (Skylar, Hong *et al.* 2010). One of the *AGRONAUTE* (*AGO*) *(D'Agostino, Deruère et al. 2000)* gene family members, named *ZWILLE* (*ZLL*), also plays a role in SAM development. *ZLL* knock-out plants show differentiation of the stem cells at a late stage of embryogenesis (Moussian, Schoof *et al.* 1998). This phenotype reveals that *ZLL* function is needed to maintain stem cells during embryogenesis via increasing the effect of *WUS* signalling from the OC to the stem cells (Tucker, Hinze *et al.* 2008). However, AGO proteins are also components of RNA interference (RNAi) pathways, which usually repress target messenger RNAs via mRNA degradation or translational inhibition. Recently, it has been shown that *AGO10/ZLL* represses microRNAs 166 and 165 (Liu, Yao *et al.* 2009). Both of these microRNAs target the same *HD-ZIP III* family genes (Jung and Park 2007). MicroRNA, miR394 was just discovered to be involved in shoot meristem maintenance. MiR394 represses a gene called *LEAF CURLING RESPONSIVENESS* (*LCR*) and when miR394 activity is inhibited in the L2 and L3 layer of the meristem centre, the meristem terminates. This termination is regulated via a yet unknown regulatory pathway, but a possibility could be that *ZLL* and miR394 pathways converge via the *HD-ZIP III* genes to regulate stem cell maintenance (Knauer, Holt *et al.* 2013).

Not only signals from the close surrounding of the organizing centre but also signals that are derived from the periphery, where the organs are initiated can have an influence on SAM maintenance. One of these is a gene called *HAIRY MERISTEM* (*HAM)* a *GRAS* transcription factor, that was first discovered in Petunia plants where mutants show a meristem arrest after the production of a few leaves (Stuurman, Jäggi *et al.* 2002). Four homologs of *HAM* present in Arabidopsis promote shoot indeterminacy and when knocked out, mutant plants develop several abnormalities including aberrant phylotaxis, altered meristem and leaf morphology and loss of shoot and indeterminacy (Engstrom, Andersen *et al.* 2011). A second signal from organs to the shoot influencing meristem maintenance, is controlled by genes expressed in an abaxial or adaxial dependent manner. Meristem arrest occurs when abaxial genes are overexpressed or adaxial genes repressed (Lin, Shuai *et al.* 2003).

The Retinoblastoma gene is associated with cell proliferation and was the first gene discovered to have tumor repressing functions in animals (Friend, Bernards *et al.* 1986). In Arabidopsis down-regulation of *RETINOBLASTOMA-RELATED* (*RBR*) leads to the differentiation and disorganization of the meristem and finally developmental arrest. It is suggested that *RBR* is required to maintain anticlinal and periclinal cell division in the L2 and L3. If *RBR* is down-regulated, *CLV3* expression becomes confined to the L1 layer leading to a transient increase in *WUS* expression (Borghi, Gutzat *et al.* 2010). It was recently shown that transient down-regulation of *RBR* leads to the collapse of the CLV-WUS feedback-loop (Borghi, Gutzat *et al.* 2010).

The extensive genetic and molecular studies in the model species Arabidopsis revealed that a complex network of many genes is required to maintain the meristematic activity and to allow differentiation of primordia into organs. Although less studied, most likely homologs of these genes exist in other plant species, for example in *Solanaceae* species, exerting a similar meristematic function and resulting also in meristem loss when mutated. One of these genes, *SELF-PRUNING* (*SP*), is assumed to be linked to the determinant versus indeterminate growth habit in tomato and also in pepper (*Capsicum annuum*). *SP* is also involved in the maintenance of the, for tomato and pepper typical sympodial growth, is *SP*. The *sp* mutant plants terminate their growth early, showing that the gene is needed for maintenance of the meristem (Pnueli, Carmel-Goren *et al.* 1998).

Environmental influence on meristem maintenance

It has been believed for a long time that leaf initiation and meristem maintenance are autonomous mechanisms controlled by only internal factors. Recently it has been shown that cytokinin mediates light responses of the SAM and that light is needed for normal cytokinin signalling in tomato (Yoshida, Mandel *et al.* 2011). When tomato shoot apices are grown in the dark, organ primordia formation is inhibited. This inhibition is independent of photosynthesis, suggesting that light acts as a signal to increase cytokinin response and therefore promoting organ formation (Yoshida, Mandel *et al.* 2011). Additionally auxin might be influenced by light too, because in dark exposed apices the PIN1 protein was gradually internalized and lost from the plasma membrane (Yoshida, Mandel *et al.* 2011). Also other studies have reported an influence of the environment on the maintenance of meristems in plants. The phenomenon of meristem defects, also known as '*blindness*' were already described in 1934 when the first scientific articles were published about *Brassica oleracea* plants that lose their meristematic activity (Hubbell 1934). Despite these early reports, only few studies have been published about the nature of this problem. Though, it seems to be clear that the environmental conditions under which the plants grow have a major effect on SAM arrest. Next to Brassica, spontaneous meristem loss is known in a wide range of plant species, which is why probably a general mechanism is underlying this phenomenon. It has been reported in e.g. rose (*Rosa hybrida L.*), baby's breath (*Gypsophila paniculata*), tomato (*Solanum lycopersicum*) brassicas (*Brassica oleracea var.italica, Brassica oleracea convar. botrytis var. botrytis, Brassica oleracea convar. gongylodes*) (Wurr, Hambidge *et al.* 1996) and bell pepper (*Capsicum annuum*) (van der Burg 1999). Examples of blind brassica seedlings are shown in Figure 4.

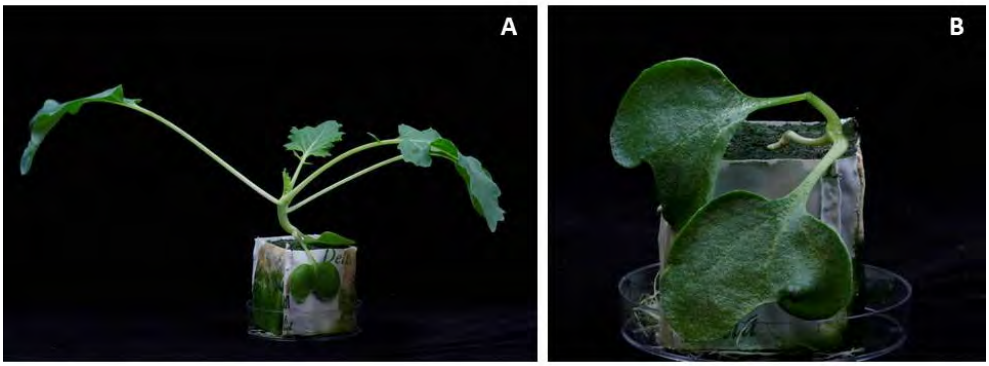

Figure 4: Blind Brassica seedling. A: Normal four week old kohlrabi seedling B: Blind four week old kohlrabi seedling with arrested meristem.

The missing SAM phenotype in *Brassica oleracea* plants is explained as the abortion, or differentiation of shoot apical meristem cells and not as a necrosis of the tissue (Wurr, Hambidge *et al.* 1996; Forsyth, Pearson *et al.* 1999b). This observation suggests that the molecular network maintaining the stem cells is disrupted and therefore, cells differentiate. This failure leads to cessation of further plant development. Studies conducted to unravel the conditions leading to SAM arrest in Brassicas come up with several environmental cues:

Low solar radiation (Wurr, Hambidge *et al.* 1996), low temperatures during very early stages of plant growth (Wiebosch 1950; Smith 1953), combination of both conditions, low light and low temperature (Forsyth, Pearson *et al.* 1999b), a period of drought after planting in the field (Wiebosch 1950), nutrition e.g. too rich in nitrogen (Wiebosch 1950) or molybdenum deficient (Neenan and Goodman 1954).

To solve the problem of sudden occurrence of blind plants, it is important to unravel which environmental cues could lead to the arrest of the meristem in brassica species especially because the production losses can reach up to 96% in broccoli and 60% in cauliflower (Smith 1953). The demand for *Brassica oleracea* plantlets during early spring to avoid the so called “white weeks” in which the prices for cauliflower drop tremendously, forces plant grower to sow seeds in the winter and raise plantlets in the greenhouse in order to plant them out into the fields during late winter. Especially those growth conditions are thought to be associated with the occurrence of the phenomenon of SAM arrest. However, no real proof

about the exact conditions that cause blindness has been found until today, partly because the occurrence of the phenomenon varies a lot between years, probably due to difference in climate, and its dependency on genotype and seed lot. Salter and co-workers treated a cauliflower variety at the 7th leaf stage for 7 days at low temperatures (0.5°C) and obtained high levels of plants with arrested meristems (Salter 1957). In the model plant *Arabidopsis* thaliana *crn-1* mutants show more severe stem fasciation due to aberrations of the SAM at 29°C than when grown at 22°C. This suggests that in *crn-1* the development of the SAM becomes temperature sensitive, which could be an indication that indeed temperature can have an effect on the meristem (Müller, Bleckmann *et al.* 2008). Additionally, it has been demonstrated that the expression of the auxin synthesis gene YUC8 is elevated at 29°C leading to higher free IAA levels in planta, showing that genes involved in the auxin production pathway are influenced by temperature and therefore could play a role in temperature dependent SAM arrest (Sun, Qi *et al.* 2012).

SAM loss in brassica, tomato and bell-pepper

Recently, a study was published where aberrant phenotypes of brassica were analysed (Chable, Rival *et al.* 2008). In that study their final hypothesis was that aberrant phenotypes develop in brassica due to epigenetic changes, especially DNA methylation of cytosine residues. Taken into account that only a certain percentage of a genotype arrests their meristem under certain unknown environmental conditions, this hypothesis could very well be true.

In brassica the termination of meristematic activity can occur at a young stage, directly after germination or at later stages of development when the plants have already developed up to ten leaves (Salter 1957)(Forsyth, Pearson *et al.* 1999b).

Similarly to brassicas, blindness in tomato plants can occur at different developmental stages. Blind tomato plants appear after pruning and grafting of plantlets. These plants develop only one or no axilary shoots from the cotyledon axils (Figure 5 B and C).

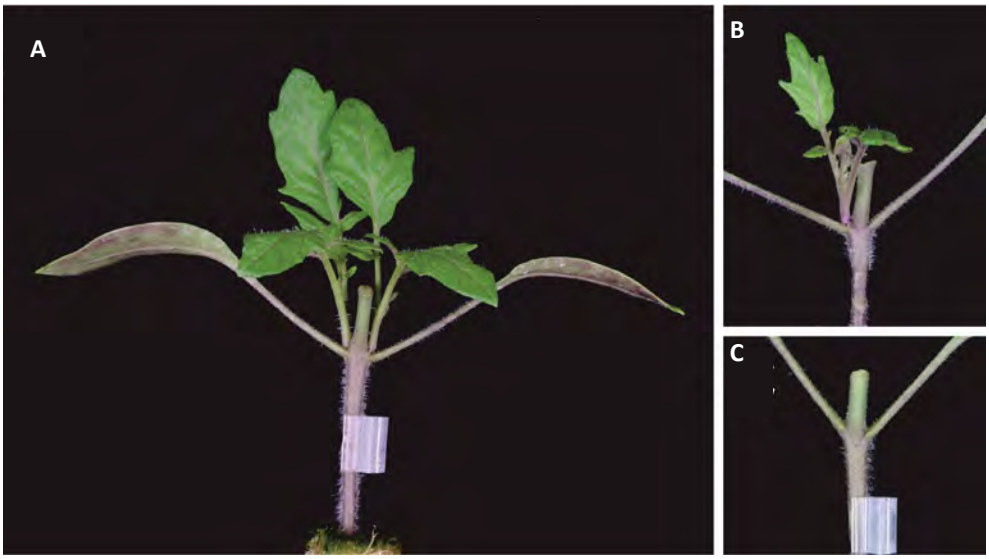

Figure 5. The three different tomato phenotypes that can occur after pruning. A) normal grafted tomato plant where both axillary meristems grow out and develop into two new shoots. B) grafted tomato plant where one meristem is arrested and only one of the two shoots develops. C) grafted tomato plant where both of the axillary buds are arrested and never form a shoot.

Literature about the blind problem in tomato is very limited. Nevertheless, several genes involved in meristem maintenance have been identified in tomato, amongst others the *WUS* homolog (Wang, Wang *et al.* 2012). Furthermore genes related to meristem maintenance have been analysed in tomato, such as a gene called "blind". In the absence of this gene, which encodes a MYB transcription factor, plant exhibit an almost perfect resemblance of the phenotype of the lateral suppressor (ls) mutant which controls the axiliary meristem outgrowth (Schumacher, Schmitt *et al.* 1999) and (Schmitz, Tillmann *et al.* 2002). Another gene is called Defective Embryo and Meristem (Leibfried, To *et al.* 2005). In the absence of this gene, plants have small slightly abnormal cotyledons and no SAM (Keddie, Carroll *et al.* 1998). The *GOBLET* (*GOB*) genes is important for SAM maintenance and mutations in the *EXPELLED SHOOT* (*EXP*) gene leads to termination of the SAM after the development of only a few leaves (Brand, Shirding *et al.* 2007). However, the exact roles of these genes and the pathways in which they are involved are not well studied in tomato. We assume that the pathways for meristem maintenance are somewhat similar to the ones in Arabidopsis. *Capsicum aestivum* (bell pepper) also suffers from the blindness phenomenon and the problem is well-known among plant-raisers,

growers and breeding companies. However, arrested seedlings develop under certain environmental conditions only, and it has been shown that the frequency of pepper seedlings with arrested SAM increases when grown under a combination of high temperature and low light intensity conditions (van der Burg 1999). In order to prevent the arrest of bell pepper plants it has been advised to decrease the number of hours of light after potting until the plants have rooted equally (Brakeboer 2008). The blindness phenomenon is apparently influenced by genetic susceptibility as well, which is based on the observation that some varieties develop more blind seedlings than others under the same environmental conditions. Nevertheless, the environment plays a major role in the expression of the arrest because susceptible varieties produce different levels of blindness at different growing locations (van der Burg 1999).

Inventory of knowledge from experts about the occurrence of blindness

Although the phenomenon of SAM arrest is known in brassica for more than 50 years, only a few studies have been published on this topic. Therefore, we decided to interview specialists in the field to learn more from their experience with SAM arrest and to avoid performing experiments that have been done already, but that were not published. We interviewed employees of breeding companies, plantlet producers, and seed technology companies in the Netherlands. In these interviews, the experts were especially questioned about susceptible varieties and environmental conditions that could trigger SAM arrest in brassica and tomato. In both tomato and brassica, temperature was mentioned as a suspected factor for the occurrence of this phenomenon. However, while high temperature was mentioned to cause SAM arrest in tomato, it was suggested to be low temperatures in brassica. Besides temperature the experts also stated that light intensities and the imbalance of light and temperature could be causing SAM arrest in tomato. Therefore, we aimed to focus in our studies on temperature and light intensities and its effect on the maintenance of the SAM in tomato and brassica.

Table 1. Summary of factors that could cause SAM arrest based on interviews with several experts in the field of tomato and Brassica breeding and production.

Tomato	Brassica
Stress	Leaf temperature below 8 °C
High light intensities	Low temperatures during the night at 5^{th}-6^{th} leaf stage
Temperatures above 25 °C and low light intensity	Germination at low temperature
High light intensities and low temperature	Change in temperature from high to low during vegetative growth stage
Temperature fluctuations	Low temperatures and low light intensities
Seed-priming	
Damage of cotyledons	
Cultivar	
Low light intensities and low temperatures	

Aim and outline of the thesis

The aims of the research described in this thesis were to unravel the environmental conditions that can influence the maintenance of the shoot apical meristem in brassica and tomato and to understand if there is a genetic basis for SAM arrest in both species. Many molecular components that build the network underlying meristem maintenance are known in *Arabidopsis thaliana*. However, in contrast to Arabidopsis, brassica and tomato plants suffer from spontaneous SAM arrest due to unknown environmental conditions. This gives opportunities to study the influence of the environment on meristem maintenance. The results obtained in this thesis open new avenues for future research in a so far rather unknown area of research.

The main research questions addressed in this thesis are:

1. Which environmental conditions lead to SAM arrest in tomato?
2. Which environmental conditions lead to SAM arrest in brassica?
3. Is there a genetic component leading to the sensitivity to environmental conditions that triggers SAM arrest?
4. Can the sensitivity be overcome?

Chapter 1, the general introduction describes the scientific background about SAM maintenance in the model species *A.thaliana*, highlights what is known about spontaneous SAM arrest in different plant species and the knowledge that experts in the fields of plant production and plant breeding have about causes of SAM arrest in tomato and brassica.

Chapter 2 describes the identification of the environmental and genetic component that leads to SAM arrest in *Brassica oleracea*. Low temperatures during germination induce SAM arrest in susceptible varieties. One region on chromosome three is associated with the sensitivity and in this region over 300 genes are located. A transcriptome analysis empowered us to minimize the potential candidate genes in this region to 39. We found that cyclins are differentially expressed in sensitive genotypes compared to resistant ones. However, nuclei in the meristem do not pass through the s-phase in sensitive genotypes compared to resistant ones and this seems to be specific for nuclei in the meristem region.

Chapter 3 shows the discovery of a seed treatment that prevents the development of SAM arrest in sensitive brassica genotypes. Pre-imbibition for two hours prior to the cold treatment prevents SAM arrest. Seeds can be dried back to initial seed moisture content, maintaining this resistant effect only if they are imbibed at certain moisture content. Increasing the moisture content can re-induce susceptibility.

Chapter 4 is designated to the morphological description of SAM arrest in tomato and the identification of environmental conditions causing SAM arrest in tomato. The study also identifies the sensitive period in which adverse growing conditions

induce the arrest. Light intensities of 600 µmol compared to 100 µmol induce SAM arrest, likely mediated through an irradiation induce increase in temperature at the seed level, since also a high temperature of 33°C compared to 19°C can strongly stimulate the development of blind plants. We were able to show that the sensitivity for temperature is strongest the first 48h after starting imbibition.

Chapter 5 discusses the outcomes of this thesis and suggests future experiments to unravel genetic and physiological aspects of the blindness phenomenon in more detail.

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Temperature affects meristem maintenance in *Brassica oleraceae*

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Chapter 2

Abstract

Most of a the above ground tissues in higher plants originate from a pool of cells known as stem cells, which are located in the centre of the growing tip. Brassica oleracea plants can suffer from spontaneous arrest of the stem cells in the shoot apical meristem (SAM), resulting in so-called blind plants. The unpredictable occurrence of blind plants can lead to considerable economic losses. Analysis of the environmental factors causing this phenomenon showed that low temperature during germination can induce SAM arrest in susceptible seeds. A quantitative genetic mapping approach identified a region on chromosome three, containing over 300 genes, as associated with blindness sensitivity. A transcriptome analysis reduced the number of candidate genes in this region to 39. Among these candidate genes is a homolog of the *MCM2-3-5* genes known to be involved in DNA replication and particularly essential in fast dividing cells. Under inducing conditions cyclin genes are expressed at a lower level in seedlings from sensitive genotypes compared to resistant ones.

Introduction

Plants are sessile organisms that have to adapt to the conditions of their environment (Ruts, Matsubara *et al.* 2012). This environment varies regularly, ranging from optimal conditions for plant growth and development towards abiotic or biotic stresses (Janská, Maršík *et al.* 2010). Moreover, the shoot and root of a plant, containing the stem cells responsible for growth, are exposed to different environmental conditions at the same moment and therefore may respond differently. Plants have the ability to adapt to abiotic stress (e.g. temperature, drought and salt) via specific signalling pathways involving phytohormones and regulatory proteins, such as receptors, protein modifiers and transcription factors. Responding to external factors is part of normal plant development, for instance a switch from vegetative growth to generative growth (Kaufmann, Pajoro *et al.* 2010). These processes are controlled in part by environmental signals that are perceived by the plant and internally processed leading to changes in gene expression (Koornneef, Hanhart *et al.* 1991; Davis 2009). Upon germination and during the vegetative stage of development, a small and almost constant number of pluripotent stem cells is located at the shoot apical meristem (Yanai, Shani *et al.* 2005) and the root apical meristem (Van Zanten, Koini *et al.* 2011). These stem cells are renewed throughout plant development and part of the cells in the shoot apical meristem (SEM) differentiate to form the aerial parts of a plant (Barton and Poethig 1993). The SAM can be divided into a central zone that is essential for maintenance of the meristem and a peripheral zone, from which lateral organs are initiated (Jürgens 1995). In the plant model species *Arabidopsis thaliana*, the maintenance and differentiation of central stem cells, is organized by a feedback loop between *WUSCHEL*, a homeobox transcription factor, and *CLAVATA* genes (Laux, Mayer *et al.* 1996 (Schoof, 2000 #25).

When the balance between maintenance of stem cells and cell differentiation is distorted plants can lose apical meristem function, as has been described for a range of plant species, such as tomato (*Solanum lycopersicum*) (Wetzstein and Vavrina 2002), baby's breath (*Gypsophila paniculata*) (Hicklenton, Newman *et al.* 1993) and brassica (*Brassica oleracea* crops such as cauliflower and broccoli) (Wiebosch 1950). In general, unfavourable environmental conditions are believed to cause

apical meristem loss in sensitive plants. In brassica this phenomena is known as "blindness" and prevents the production of a marketable broccoli or cauliflower head. Blindness in brassica is characterized by a termination of leaf primordia production by the SAM (Forsyth, Pearson *et al.* 1999b). It has been reported that depending on the developmental stage of the plant and the moment of exposure to the inductive conditions, five to ten leaves can be formed before the SAM ceases (Wiebosch 1950). In these cases, the last formed leaves can either be of normal shape or consist of a petiole only, without a leaf blade (Forsyth, Pearson *et al.* 1999b). Wiebosch *et al.* (1950) distinguished between three forms of blindness: empty hearted plants (with a dent in the stem), needle types (forming a pin like structure at the position of the SAM) and pitcher plants (that form as last structure a pitcher shaped leaf). For plant growers it is especially problematic, because recognizing affected plants before transplanting them into the fields is hardly possible, which leads to high economic losses that can be up to 95% in broccoli (Hambridge 1993). The phenomenon of blind plants has been described already in the 1940s. During 70 years of research, aiming to identify potential causes, various environmental factors have been proposed to induce blindness: Low solar radiation (Wurr, Hambidge *et al.* 1996), low temperature during early stages of development (Salter 1957), freezing conditions (Mounsey-Wood 1957), molybdenum deficiency (Agarwala 1952) and sowing date (Wurr, Hambidge *et al.* 1996). Forsyth *et al.* (1999) concluded that the apical region of broccoli has a high degree of frost tolerance and that freezing alone is not sufficient for apical abortion. To study and elucidate the basis of SAM arrest in brassica, it is essential to have a reproducible induction system for blindness under controlled conditions. The induction method developed by Wurr *et al.* (1996) is time and space consuming as blind plants develop several leaves before losing SAM activity. Additionally, this system seems to be suitable for broccoli (*B. oleracea convar. botrytis*) only. Therefore, we aimed to establish an early blindness induction system, useful for a broad spectrum of *B. oleracea* crops and to apply this method to study the morphology, physiology and molecular mechanisms underlying SAM arrest in brassica plants.

In this paper, we present the morphological behaviour of SAM cells from blindness susceptible and resistant genotypes. An assay was developed to induce reproducibly SAM abortion (blindness) in susceptible *B. oleracea* genotypes at seedling stage. This assay can be used by breeding companies to screen their germplasm and seed-lots for susceptibility to blindness induction at an early stage and provides a tool to study the underlying physiological and molecular causes of this phenomenon. The assay was used to investigate the expression patterns of key genes involved in SAM maintenance during the initiation of blindness in susceptible genotypes. Furthermore, the blindness inductive system was implemented in a genetic analysis, which enabled the identification of a genetic region associated with blindness in *B. oleracea*. Comparing the transcriptome of a resistant and sensitive line showed that several genes involved in the cell cycle are differentially expressed. Additionally, EdU staining, a marker for cell cycle activity, showed lower signals in nuclei located in the SAM of the sensitive genotype compared to nuclei in the SAM of a resistant line.

Results

SAM defects in Brassica oleracea seedlings

During normal seedling growth, *B. oleracea* plants successively and continuously produce leaves. We analysed the phenotype of normal and blind brassica plants in more detail and observed variation in the number of leaves and aberrations in architecture. For this purpose, blindness was studied in a sensitive variety 'Stanton'. Plants of four weeks old were macroscopically analysed and an example of a normal and blind plant is depicted in Figure 1. The normal young brassica plant had developed three leaves, next to two small developing leaves emerging from the apex (Figure 1A and B). The blind brassica plants of the same age developed two leaves and stopped the production of more leaves (Fig 1C and D). Although the overall morphology of the two types of plants is very similar (in size and leaf development) at this particular stage, further development of the blind plant is arrested. At the position where new leaves emerge in the normal plant, the arrested plant showed a dent and exhibited the absence of leaf development and growing tip (Figure 1D). We observed that the stage of development at which the

arrest of the meristem occurs, is variable and blind plants exhibiting this phenotype can vary in their appearance from no leaves to plants with a few leaves, although these leaves are often aberrantly shaped (Figure S1 D Chapter 2). Some arrested brassica seedlings have the ability to generate new meristems from the axils of their cotyledons (Figure S1 A Chapter 2) and these axillary meristems can develop into a proper shoot that continues development.

Figure 1.
Phenotype of a normal *B. oleracea* plant compared to a plant of the same genotype with spontaneously arrested shoot apical meristem (Yanai, Shani et al. 2005). A and B Four weeks old normal *B. oleracea* plant grown in soil. C and D: a plant sown at the same time of the same genotype treated in the same way but with arrested shoot.

Brassica oleracea plants exhibiting shoot apical meristem arrest have disorganized shoot apical meristems

A normal shoot apical meristem can be subdivided into three zones: the central zone, the periphery zone and the rib zone (Jürgens 1995). To examine more closely the SAM region of blind plants, we conducted histological analysis of the SAM area from normal and blind seedlings. The sections of six-day old normal seedlings show all three zones of a typical SAM (Figure 2). The central zone comprises the stem cells, which stay pluripotent throughout a plants life and this region is detectable as a group of small cells. It is easy to identify the rib-zone by the parallel file structure of cells (Figure 2). In the centre of the cotyledons and the main stem the vasculature can be seen. This tissue is also visible in the sections of the blind seedling, indicating that the sections were made approximately at the same position of the seedling. In comparison to the tunica-corpus structure present in normal seedlings, blind seedlings display an aberrant or absent structure of the three typical zones in the SAM (Figure 2 C and D). Additionally, the regular cell files below the rib-zone in normal plants (Figure 2B) are absent in the arrested seedlings and show an irregular pattern (Figure 2D). In general the patterning of the cells is less organized and most of the fast dividing and small meristematic cells present in a normal SAM (Figure 2B, CZ) are replaced by larger and less organized parenchyma-like cells. In the analysed seedling no leaves were formed and no leaf primordia are visible (Figure 2D).

To analyse the phenotype at an earlier stage we performed scanning electron microscopy (SEM)(Figure 3). In the SEM pictures (Figure 3 A and B) we observed a dome shaped meristem from a normal seedling with an emerging leaf primordium at the flank of the dome, while the blind seedling show a flattened meristem with only one leaf-like structure emerging (Figure 3 C and D).

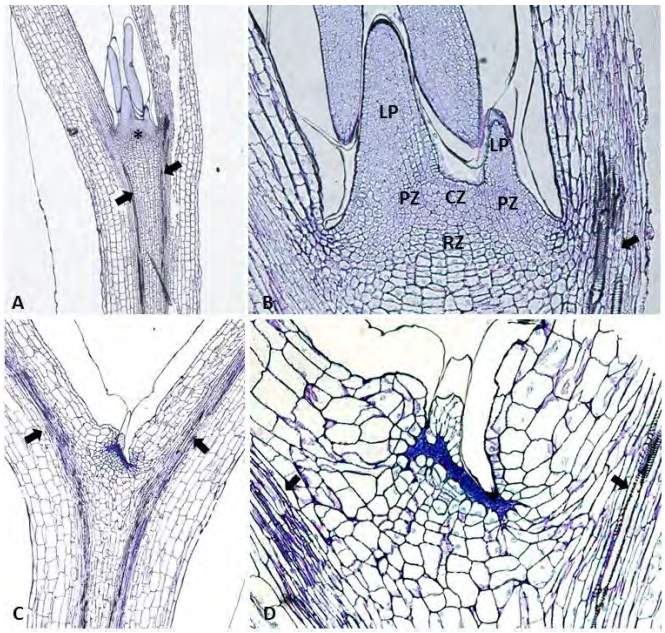

Figure 2.
A: Histological differences *B. oleracea* seedling with a normal (A and B) or an arrested meristem (C and D). Stars indicate the region of the meristem and arrows point at the vascular tissue reaching into the cotyledons. CZ: central zone; LP: leaf primordium; PZ: peripheral zone; RZ: rib zone.

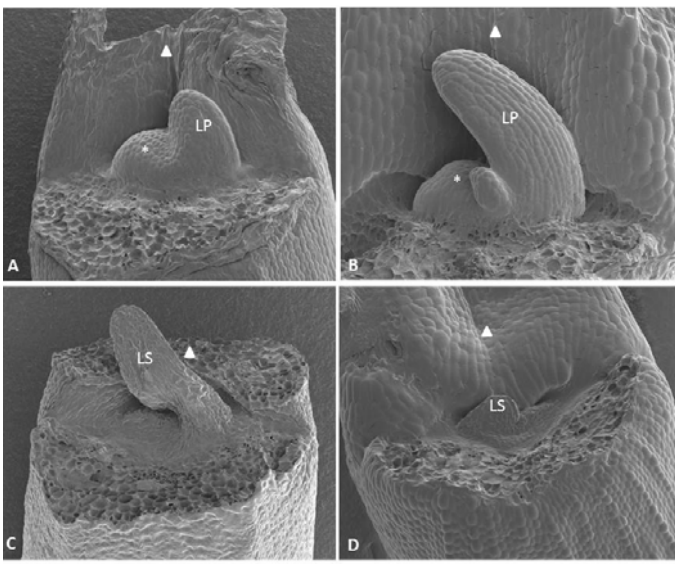

Figure 3.
The shoot apical meristem in *B. oleracea* seedlings from cultivar 'Stanton', which is susceptible to meristem arrest. A: Three days old seedlings with a normal SAM and one leaf developing. B: Four days old seedling with one leaf emerging and normal SAM. C: Three days old seedling with an aberrant SAM and one leaf-like structure. D: Four days old seedling with an aberrant meristem. Asterisks indicate the meristem, arrow heads indicate the cotyledons and LS: leaf like structures, LP: marks the leaf primordium.

Blind shoot is associated with blind root

While comparing the germination behaviour of sensitive genotypes to resistant ones, we observed that some of the blind seedlings not only lost their SAM but also their root meristematic activity (Figure 4). The average root length of seedlings with

arrested meristems during a seven-day period is given in Figure 4. The first time-point with a significant difference (p-value < 0.05) in average root length was at day three. At day ten, all blind plants had a root length of three to four centimetres while normal plants had an average root length of seven to eight centimetres. Furthermore, some of the blind plants did not show a macroscopically visible root meristem and never developed further (Figure 4H). Investigation of germination efficiency revealed that the difference in root length is not due to a lower germination speed, because germination occurs at the same time independently of the phenotype. Based on these results we can conclude that arrest of the SAM does not affected germination capacity and root growth might develop either during or after germination.

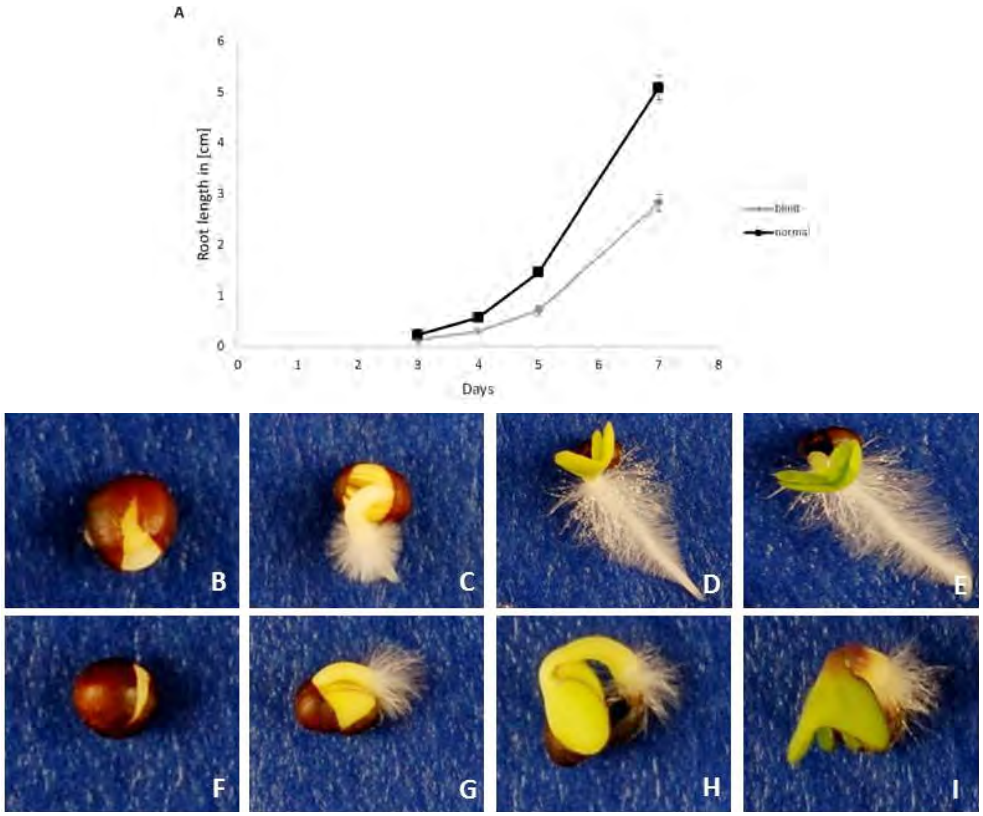

Figure 4. Root development of seedlings showing a blind and a normal phenotype. A: Average root growth in cm during seven days of seedling development with blind and normal seedlings grown in vertical plates on agar. Standard deviation is given for n= 50 seedlings of three replicates. Representative pictures of normal seedlings (B-E) and seedlings with shoot apical meristem arrest (F-I), on day 1 (B, F), day 2 (C, G), day 3 (D,H) or day 4 (E, I) after transfer from the cold induction treatment to 20 °C.

Blindness inducing assay

The environment plays an important role in plant development and can influence plant growth positively or negatively (Janská, Maršík *et al.* 2010). Since cold was found to induce blindness in brassica (Salter 1957), we investigated the role and effect of low (non-freezing) temperatures on SAM loss during early brassica plant development. To induce blindness, seeds of a sensitive seed lot were imbibed at temperatures ranging from 0.7°C to 10.5°C for one to fourteen days (Figure 5). A clear correlation between severity of blindness and length of the treatment was observed. At the lowest temperature and longest cold imbibition-time, the highest frequency of blind seedlings was obtained. Incubation around 0-1°C proved to be the best induction temperature for an assay to analyse sensitivity and induce the blindness phenomenon. Extending this induction period further to 15 or 20 days did not significantly increase the frequency of blind plants (data not shown), but it did decrease the number of germinating seeds. After ten days of exposing seeds to cold incubation, none had reached the phase of radicle protrusion, which occurred within 24hours after transfer of the seeds from the cold to 20 °C.

To test whether there is a general mechanism involved and if the assay developed here can be widely used to screen a range of *B. oleracea* seed lots and genotypes, we tested also seeds from a kohlrabi and a broccoli variety used for vegetable production (Figure 6). Seeds from all three varieties developed blind seedlings after exposure to the inductive treatment, but with varying frequencies suggesting differences in sensitivity for the treatment or for developing blindness in a certain seed lot or genotype. The kohlrabi seeds showed the highest frequencies of blindness (65%) and broccoli seeds the lowest (15%) (Figure 6). Without the blindness induction treatment all seedlings developed normally, i.e. containing a functional SAM. These results show that our induction method is broadly applicable among *B. oleracea* varieties, while the variation suggests the potential involvement of genetic components.

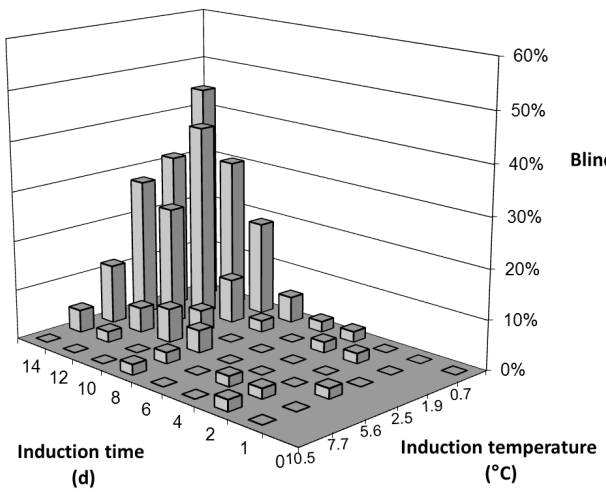

Figure 5.
Effect of temperature and induction time on the frequency of *B. oleracea* plants with arrested shoot apical meristem. Seeds of the variety "Stanton" (white cabbage) were imbibed for different durations at various temperatures, followed by transfer to 20 ˚C.

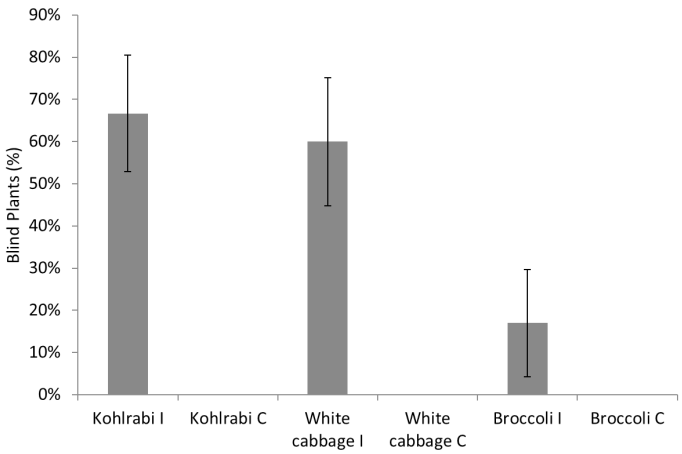

Figure 6.
The frequency of shoot apical meristem arrested plants obtained from three seed lots representing three *B. oleracea* morphotypes after exposure of the seeds to the blindness inducting conditions (I) or directly sown at 20 ˚C as control condition (C). Data are the results of the percentage of blind plants with standard error of two independent experiments using 53 seeds each.

2

Chromosome three contains a region associated with shoot apical meristem arrest in Brassica oleracea

To investigate the genetic mechanism regulating SAM loss we analysed a *B. oleracea* mapping population (AGDH population (Sebastian, 2000 #48)) comprising of 100 doubled haploid lines established from a cross between the variety 'Green Duke' of which a double haploid (GGDH) was made, and a rapid cycling brassica. Using our blindness test described above, we determined that the sensitivity for blindness varied significantly between the two parents. The susceptible parent is the broccoli

(GGDH), which responded to the induction system by losing the meristem. In the AGDH mapping population fully resistant lines were present that developed normally and never lost the SAM after the blindness assay, as well as lines that developed a high frequency of blind seedlings in response to the cold treatment (Figure 7). This result confirms the previous indication of the involvement of a genetic factor, which was obtained after testing seed lots of different *B. oleracea* morphotypes. The sensitivity of some of the offspring lines in the AGDH population is even higher than that of the parents (Figure 7), which means that there is positive transgression of this trait. For example, seedlings from line AG1020 never developed blind plants after the cold induction, whereas seedlings from the most susceptible line AG5010 produced 55% blind plants. These two contrasting lines are used in further gene expression studies described below. QTL mapping of the sensitivity trait to blindness resulted in one significant QTL on linkage group three, with the most significant region spanning from 83.1 to 84.5 cM (p-value 0.001) (Figure S2). This significant QTL explains 24% of the total variance. The QTL analysis was repeated with three subsequent seed-productions of the AGDH population and all three indicating a major QTL on the same region of chromosome three.

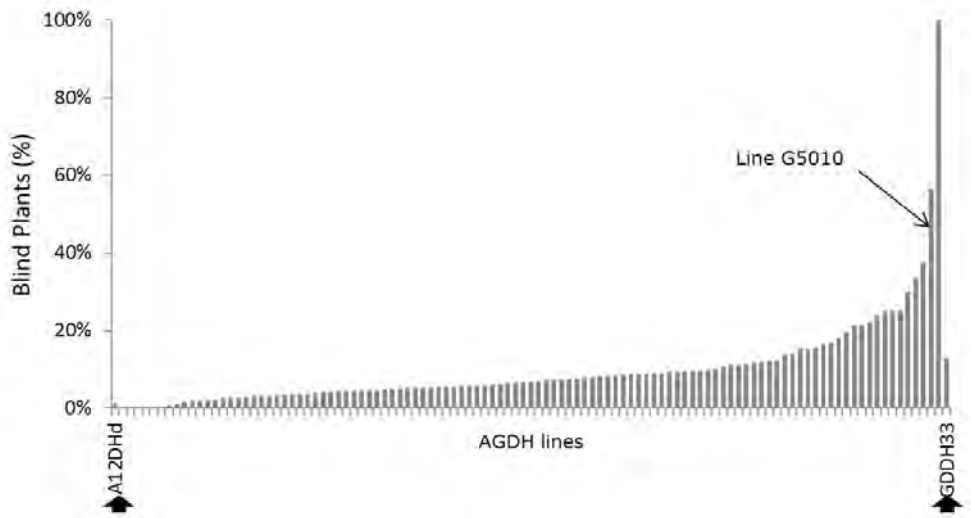

Figure 7. Genetic variance of sensitivity to blindness induction. Seeds of the AGDH population were exposed to blindness inducing conditions of 10 days at 0.3^0 C, scored for the percentage of blind plants. Arrows point to the parental lines of the population. Results represent the mean percentage of blind plants from three replicates (n= 20 seedlings).

Shoot Meristemless is lower expressed in blind plants

To get an indication at what time-point after the cold induction the SAM loses its meristematic cells, gene expression studies were conducted in the homozygous lines of the AGDH population that showed high frequencies of blindness (sensitive) and no blind seedlings after the cold induction. The expression was measured of a gene important for meristem maintenance and which is broadly expressed throughout the meristem. Such a gene is *SHOOT MERISTEMLESS (STM)*, encoding a class-I KNOTTED-like homeobox transcription factor that is expressed throughout the non-organogenic regions of the SAM, but is excluded from regions of leaf primordia formation (Long, Moan *et al.* 1996; Rupp, Frank *et al.* 1999; Scofield, Dewitte *et al.* 2007). In *Arabidopsis thaliana, STM,* has a role in preventing stem cell differentiation (Lenhard, Jürgens *et al.* 2002) (Williams and Fletcher 2005). A homolog of this gene was already identified in *B. oleracea* (http://www.ncbi.nlm.nih.gov/nucleotide/22023961?report=genbank&log$=nuclalign&blast_rank=13&RID=04GHDH7U015). Using quantitative RT-PCR, we determined that the expression of *BoSTM* is much lower in sensitive blind seedlings at day five after the cold induction compared to sensitive normal plants or the resistant line (Figure 8). This supports our finding that the meristem differentiates and less meristematic cells are present. Additionally, we measured the expression of *BoSTM* in ten day old seedlings after the cold induction in a sensitive genotype showing normal and different blind phenotypes. We found that *BoSTM* is down-regulated in all seedlings independent of their blind phenotype, which supporting our finding that the SAM is absent or that the cells in the SAM have lost their meristematic identity.

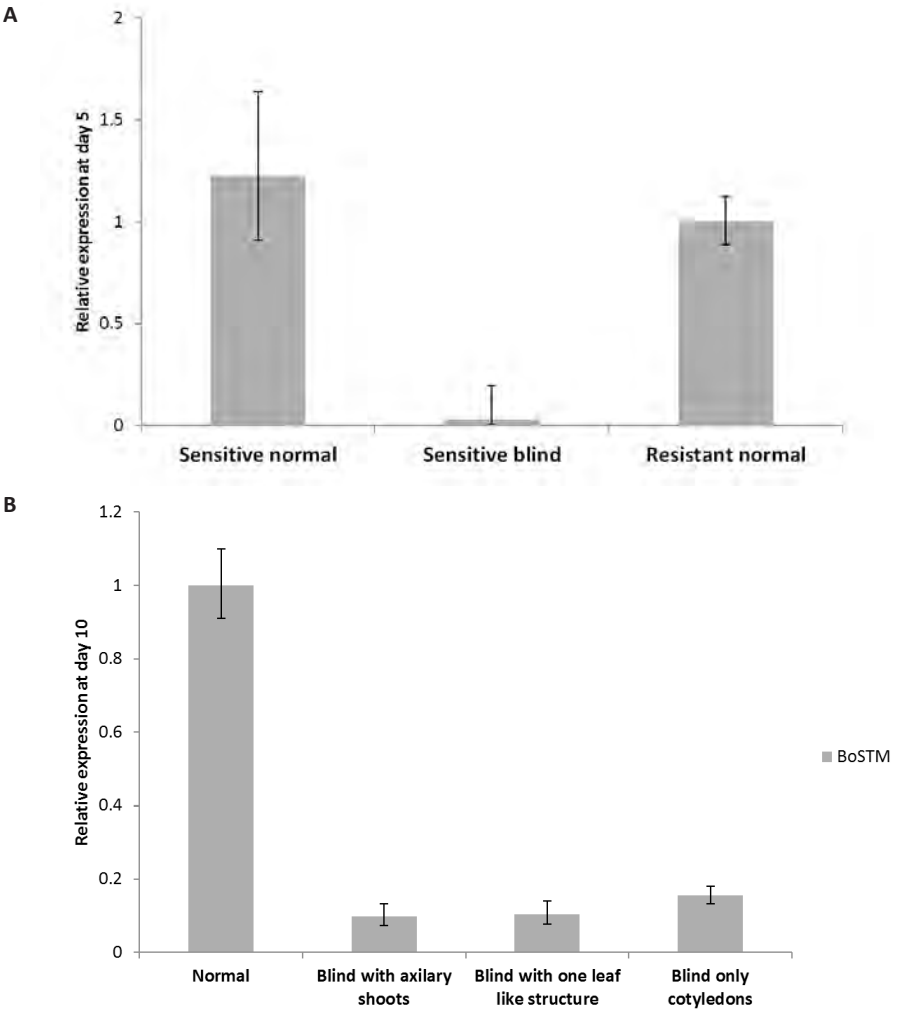

Figure 8. Relative expression of *BoSTM* (orthologous to *STM* in *Arabidopsis thaliana*) at day five after the cold induction treatment. Expression levels measured by Q-PCR using normal-looking plants (Sensitive normal) and blind-like plants (Sensitive blind) from a sensitive genotype and a resistant genotype containing normal meristems (Resistant normal). Error bars represent the +- standard error of four biological replicates. B: Relative expression of *BoSTM* at day ten after the cold induction of a sensitive genotype divided into its different phenotypes. 'Normal' represents the expression of plants with a meristem. 'Blind with axillary shoots' represents plants that lost their SAM but develop side shoots in the axils of the cotyledons. Blind with leaf like structure represent the expression of plants that produced one leaf-like structure before arresting the meristem. Blind only cotyledons, represents plants that only developed cotyledons and have a completely arrested SAM. Error bars represent the standard error of four biological replicates.

Synteny mapping for the chromosome 3 QTL region

As *B.oleracea*, the model plant *A. thaliana* is a member of the Brassicaceae family (Cavell, Lydiate *et al.* 1998). The chromosomal region of *B. oleracea* associated with sensitivity to blindness is on linkage group three between the markers pN148e and pW188b. The common ancestor of *A. thaliana* and *B. oleracea* diverged only five million years ago (Parkin, Gulden *et al.* 2005). Because the genome sequence from *B. oleracea* is unavailable, we analysed the gene content of the same region in *A. thaliana*, which could provide information about the genes present in the QTL region. Both markers spanning the region of our significant QTL are located on Arabidopsis chromosome three. The similarity of the genomes in the region of the QTL is high between Arabidopsis and the brassica species *B. napus* (Parkin, Gulden *et al.* 2005). Also the marker content and order at this region of the chromosome is conserved between *A. thaliana* and *B.napus,* which is an amphidiploid species formed by the ancestors of the diploids *B.rapa* (A genome donor) and *B.oleracea* (C genome donor). Because over 4000 genes are located on the homologous QTL region in the Arabidopsis genome, we suggest that a comparable number is present between the markers in the *B. oleracea* genome. To analyse the transcriptional behaviour of these ~4000 genes and identify possible candidate genes, involved in the blindness phenotype, we conducted a transcriptome sequencing experiment.

Transcriptional differences in blind and non-blind brassica plants

To further decrease the number of potential candidate genes in the QTL region and to identify potential causal genes, we compared the transcriptomes of two contrasting lines from the AGDH population at two different time-points with and without a cold induction treatment. Based on the expression levels of *BoSTM* (Figure 8), which is down-regulated five days after the cold induction, we selected day two after the cold induction as first time-point, to be able to identify primary targets that might initiate SAM arrest. At day two RNA samples are a mix of normal and blind seedlings, because at that moment plants are too small to determine their phenotype. To be sure to have pure samples of blind and normal seedlings, we also collected material at day seven after the cold induction because at that time-point it is possible to distinguish between blind and normal plants.

After RNA-sequencing and mapping the reads we filtered for genes that were differentially expressed in the region between the two markers from the QTL. We also extended our candidate gene search genome wide. To eliminate genes that are solely differentially expressed by the cold treatment, we selected those genes whose expression is changed only in the sensitive genotype and not in the resistant genotype (for detailed experimental set-up and analysis see material and methods). These filter criteria resulted in the identification of 3000 significantly differentially expressed genes (p-value < 0.05) at a genome-wide scale, from which 39 are located in the QTL region. If we further refine the list and select only the genes with a minimum fold change of log2=1.4 (arbitrary threshold) or higher, then the list decreases to eight genes (Table 1). All these eight genes are expressed in the Arabidopsis vegetative meristem and / or in the seed (during imbibition) according to the eGFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). AT3G19580 is expressed in the seed especially in the chalazal-endosperm, AT3G23920 is expressed one hour after imbibition in the dry seed and also at the mature green stage of the embryo. AT2G05520 is expressed in the whole young seedling plus in seeds imbibed for 96 hours that received a Farred light pulse, which inhibits germination. AT2G07690 and AT3G22660 are expressed in the developing embryo, while AT2G05120 is expressed during imbibition and in the dry seed. All eight genes are not regulated by cold, because their expression was not affected in the resistant genotype after application of cold stress. Only for one gene, Bra013177 no homolog in Arabidopsis could be identified. The rRNA processing gene AT3G22660 was identified twice in *B. oleracea* and these two oleracea genes - are most similar to Bra001877 and Bra023812 in *B.napus,* indicating that this gene is duplicated in both *B.oleracea* and *B.napus*. This gene is also differentially expressed when comparing samples from sensitive blind and sensitive normal seedlings at day seven. Together with AT2G05120 these are the only two genes that are in both lists (2 and 7 days, respectively) (Table 1 and 2). Most of the genes from the 'day seven' list (table 2) show comparable expression areas as the ones from day two in Arabidopsis, either in dry seeds, imbibed seeds, the embryo or the meristem in Arabidopsis, but three genes selected at day seven are expressed more broadly and in the whole seedling (AT3G20600, AT3G25470, AT2G04160).

Cell cycle and DNA synthesis

Since we expect cell division to slow down or stop in the SAM region of blind seedlings we focused on cell cycle genes in the genome-wide transcriptome data set. For cell division or mitosis DNA need to be duplicated in the S-Phase of the cell cycle. The only gene present in the QTL region (Table 1) and related to the cell cycle is a member of the Mini Chromosome *Maintenance (MCM)2-3-5* gene family. These genes play important roles in both the initiation and the elongation phase of eukaryotic DNA replication (Tye 1994). Other genes that are important for the cell cycle are cyclins, which were up-regulated two days after the cold induction in the sensitive genotype compared to the resistant one. The same set of genes showed down-regulation in the sensitive genotypes seven days after cold induction. Besides this set of cyclin genes, RETINOBLASTOMA RELATED (RBR) a plant homolog of the tumor suppressor gene identified in animals (Friend, Bernards *et al.* 1986), is also differentially expressed in the sensitive background in comparison to the resistant one, although at both time points of the analysis.

To analyse the cell division activity *in situ* and compare this to the observed gene-expression pattern in the SAM we germinated the seeds on growth medium containing EdU, a chemical that gets incorporated in the DNA during replication. EdU incorporation was visualized with a confocal microscope two days after the cold induction (Figure 9). Nuclei in the SAM of the sensitive genotype do not incorporate the chemical and are therefore not visible under the confocal microscope, while the nuclei in the surrounding cells do show fluorescence signal. This is in contrast to the nuclei in the blindness resistant genotype, where the signal is clearly visible in the SAM region.

Table 1. Genes located in the QTL region (based on the synteny with Arabidopsis) with minimum fold expression difference of log2= 1.4 between the un-induced sensitive sample and the induced-sensitive sample two days after the cold induction. The annotation of the genes in *B. napus* and *A. thaliana* plus their GO annotation from Arabidopsis are indicated.

***Brassica napus* annotation**	**Homolog in *Arabidopsis thaliana***	**GO annotation**	**Expression un-induced sensitive**	**Expression induced sensitive**	**Log2 Fold-Change**	**Significance (P-value)**
Bra001752	AT3G19580	zinc-finger protein 2	0.658451	6.32156	3.263	0.0001
Bra013177	/	/	7.11202	55.0815	2.953	7.17E-07
Bra001937	AT3G23920	beta-amylase 1	0.394096	1.68035	2.092	0.0190487
Bra013176	AT2G05520	glycine-rich protein 3	848.289	3056.73	1.849	1.98E-08
Bra013169	AT2G07690	Minichromosome maintenance (MCM2/3/5) family protein	89.0323	32.9716	-1.433	0.0001
Bra001877	AT3G22660	rRNA processing protein-related	43.6569	16.0961	-1.440	0.0001
Bra023812	AT3G22660	rRNA processing protein-related	38.3971	13.3366	-1.526	0.0001
Bra012189	AT2G05120	Nucleoporin, Nup133/Nup155-like	2.94427	0.208742	-3.818	0.0079

Table 2. Genes located in the QTL region (based on the synteny with Arabidopsis) with a minimum fold difference in expression of log2= 1.4 between the induced sensitive normal sample and the induced-sensitive blind sample seven days after the cold induction. The annotation of the genes in *B. napus* and *A. thaliana* plus their GO annotation from Arabidopsis are indicated.

***Brassica napus* annotation**	**Homolog in *Arabidopsis thaliana***	**GO annotation**	**Expression u Induced sensitive normal**	**Expression Induced sensitive blind**	**Log2 Fold-Change**	**Significance (P-value)**
Bra013177	/	/	5.170	25.423	2.298	8.17E-05
Bra001815	AT3G21150	B-box 32	2.876	9.162	1.672	0.001
Bra031301	AT3G21720	isocitrate lyase	1.641	5.145	1.648	0.009
Bra001841	AT3G21700	Ras-related small GTP-binding family protein	2.235	6.724	1.589	0.002
Bra029502	/	/	4.552	12.011	1.400	6.27E-05
Bra023812	AT3G22660	rRNA processing protein-related	24.661	9.569	-1.366	0.0004
Bra038252	AT3G20050	T-complex protein 1 alpha subunit	100.229	36.261	-1.467	0.004
/	/	/	24.059	8.645	-1.477	0.038
Bra031329	AT3G22142	Bifunctional inhibitor/ lipid-transfer protein/ seed storage 2S albumin superfamily protein	876.481	305.578	-1.520	3.01E-05
Bra013215	AT3G25520	ribosomal protein L5	2238.110	751.607	-1.574	0.006
Bra023940	AT3G20600	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	20.988	6.986	-1.587	0.0002
Bra015128	AT3G25470	bacterial hemolysin-related	15.136	4.664	-1.698	2.03E-05
Bra013212	AT2G04160	Subtilisin-like serine endopeptidase family protein	1.561	0.341	-2.197	0.0004
Bra016855	AT2G42840	protodermal factor 1	1451.170	306.669	-2.242	9.55E-12

Bra012189	AT2G05120	Nucleoporin, Nup133/ Nup155-like	2.708	0.526	-2.365	0.012
Bra034819	AT3G11680	Aluminium activated malate transporter family protein	1.533	0.076	-4.338	0.001

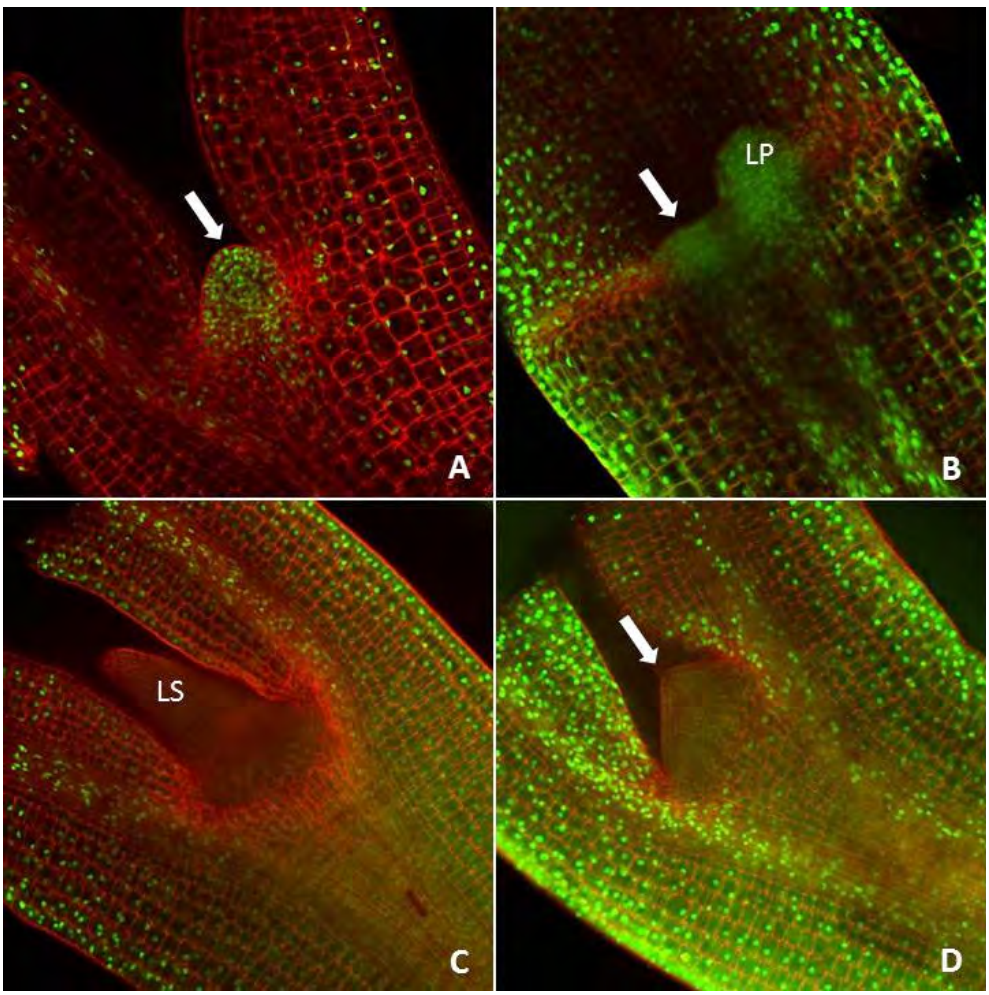

Figure 9. Meristematic region of *B.oleracea* seedlings two days after the cold treatment. The plant material was stained with EdU to visualise nuclei with DNA replication activity, indicated by green fluorescence. A and B: Representative seedlings from the blindness resistant line and C and D: Representative seedlings from the blindness sensitive line that develops SAM arrest after the cold induction. LS: Leaf like structure, arrows point at the apical dome.

Discussion

We studied the morphology and potential cause of SAM arrest in *B. oleracea* and developed a system to induce this phenomenon at a high frequency in sensitive brassica seeds. A major QTL was observed demonstrating genetic variation in sensitivity for SAM arrest induction and found that cell cycle genes are miss-expressed in sensitive genotypes after applying a treatment that induces blindness.

Blind brassica plants have an arrested SAM and this phenomenon is induced by cold imbibition

Blind seedlings show no sign of the presence of a SAM and this arrest of cell division can occur at various time-points during plant development. The phenotype expressed by the blind seedlings varies and plants can exhibit no leaves at all, a few leaves or develop leaf-like structures, while there is always a proportion of a seed batch that produces normal plants. This could be due to the fact that there is variation for the perception or processing of the signal in the meristem. Alternatively, the cold treatment gradually damages the meristematic cells in the SAM in an accumulating manner. Our histological analysis of the shoot apical meristem region showed that the cells at the position of a normal meristem of affected seedlings show characteristics of differentiated cells. They are bigger than normal meristematic cells and are not organised in cell files, suggesting that the cells have been consumed for organ formation. This is supported by sections from shoots of much older blind broccoli plants where it was found that the cells have lost their meristematic appearance as well (Forsyth, Pearson *et al.* 1999b). The normal tunica-corpus structure of the SAM (Steeves T.A. 1989; Meyerowitz 1997) was missing in the arrested seedlings, which is supporting the study by Forsyths *et al.* 1999b. Our observation that the RAMs of blind seedlings can also arrest has not been made before, although, it is known that imbibition of cauliflower seeds at low temperature reduces root growth (McCormac and Keefe 1990). More experiments are needed to analyse the correlation between RAM and SAM arrest and to understand whether both are based on the same mechanism. Blindness could be induced in susceptible genotypes when seeds were subjected to cold during the first stages of germination (imbibition). Also Forsyth et. al (1999b) found in their study that a period of cold

and low light intensities followed by a warmer period with higher light intensities can lead to blindness in broccoli. In contrast to their method, our induction system is much shorter and induces blindness in young seedlings compared to eight weeks old plants in the broccoli protocol. Additionally, our induction system proved to be working in several *B oleracea* genotypes and morphotypes, while the previously published protocol was only tested with one broccoli variety only.

Molecular regulation of SAM maintenance

The maintenance of the stem cell population, which is responsible for the production of plant organs, is controlled by a complex network of plant hormones (auxin and cytokinin), as well as a set of defined genes. The key players of the genetic framework are *WUSCHEL* (*WUS*), a homeobox gene, whose activity in the organizing centre is needed for keeping the stem cells undifferentiated and the *CLAVATA 3* (*CLV3*) gene, which is activated by WUS and in turn regulates *WUS* expression (Schoof, Lenhard *et al.* 2000). In addition to this negative feed-back loop of *WUS* and *CLV3* a class 1 knotted-like transcription factors (*SHOOT MERISTEMLESS, STM*) was identified to be responsible for embryonic SAM development and post embryonic maintenance of the SAM (Barton and Poethig 1993; Endrizzi, Moussian *et al.* 1996). The phenotype of *stm* knock-out mutants in Arabidopsis resembles the phenotype of blind brassica seedlings (Long, Moan *et al.* 1996). The expression of the homolog of *STM* in *B. oleracea* was down regulated at day five after cold induction, suggesting that the cells of the SAM are indeed differentiated and supporting the morphological changes in the arrested seedlings. Additionally, *BoSTM* was also lower expressed in blind seedlings that developed only one leaf or in blind seedlings forming side-shoots, showing that the molecular network of maintenance is affected in the different blind phenotypes. The axillary shoots observed in some blind plants developed further and remain indeterminate, taking over the function of the SAM. These axillary 'escape' shoots are also observed in Arabidopsis *wus* mutants (Laux *et al.*, 1996). This phenotype suggests that the molecular mechanism underlying the cold treatment sensitivity is specific for the cells in the SAM and that axillary buds are released from growth repression after the loss of the SAM. This is in line with the fact that axillary bud outgrowth is inhibited by the presence of the main

shoot ('apical dominance') by the plant hormone auxin and in the absence of the SAM, lateral meristem outgrowth is triggered (Cline 1994).

A genetic factor controls blindness sensitivity in brassica

The developed blindness inducing assay was applied to different brassica morphotypes, showing that this assay is applicable to a range of brassica types. However, the frequency of blind seedlings varied between the different genotypes, indicating a potential genetic component involved in the development of blindness. This let us to test a mapping population where the phenotype of the parents was contrasting and the trait blindness segregated in the F_2 offspring. With this approach we were able to identify a region on chromosome three associated with blindness sensitivity, proving that blindness sensitivity indeed is genotype dependent.

Changes in the transcriptome give clues about the molecular mechanism underlying the development of blindness

Upon low temperatures plants can change their gene expression patterns and biochemical processes to acquire tolerance to subsequent freezing (White, Simmonds *et al.* 1994). In perennials most shoot apices can cold-acclimate to survive the winter (van der Schoot and Rinne 2011). In order to induce blindness in sensitive genotypes initially dry seeds are subjected to cold imbibition. Normally imbibition should lead to germination of the seed in favourable conditions, however before germination seeds need to resume their growth and development (Bewley 1994). Germination is defined to be completed after radicle protrusion. During this process seeds reactivate several metabolic and molecular processes under which also DNA-repair, transcription, translation, directional cell expansion and finally activation of cell division in the root apical meristem and in the SAM (Bewley 1997) (Koornneef, Bentsink *et al.* 2002). In our transcriptome analysis cell cycle genes were up-regulated two days after the cold induction in sensitive genotypes compared to the resistant genotypes (Table 2). However at day seven after the cold induction the same genes all showed strongly reduced expression levels in the sensitive genotype compared to the resistant one (Table 2). This indicates that in

the sensitive genotypes the cell cycle is arrested and no new cells are formed. This is in line with the phenotype that blind seedlings develop. This arrest of the cell cycle, was also confirmed by EdU staining that marks nuclei in S-Phase, where the dye gets incorporated in DNA during replication (Schiessl, Kausika *et al.* 2012). Specifically, the nuclei in the SAM showed lower staining in the sensitive genotypes compared to the resistant ones after cold treatment. Based on this result we can conclude that the mass up-regulation of cyclin genes at day 2 in the sensitive genotype is not associated with increased DNA synthesis and cell division activity, as reflected by the EdU signal patterns in the SAM region of normal versus blind seedlings. During normal cell-cycle progression different types of cyclins show maximum expression at specific time-points of the phases of the cell cycle (G0/G1-S-G2-M). For example, while *CYCD3* peaks at the G2/M phase, *CYCA3* reaches its highest expression levels at the S-phase of the cell-cycle. Therefore, the massive up-regulation at day 2 of different types of cyclins in the sensitive genotype could still be related to the blind phenotype. It is possible that the tightly controlled expression of the cell cycle genes in normal dividing cells is lost and as a result many *CYC* genes are miss-expressed in the sensitive genotype. This could be an indication for an uncoupled cell-cycle or for the failure of the normal cell-cycle. Another major regulator of the cell cycle is RETINOPLASTOMA LIKE (RBR), which shows lower expression levels in sensitive genotypes. Recently, it has been shown that decreased *RBR* expression causes arrest of plant development and acts especially on stem cell maintenance in the SAM (Borghi, Gutzat *et al.* 2010). They showed that lack of *RBR* expression alters the meristem activity by disruption of the CLAVATA-WUSCHEL feedback loop. In a similar way the miss-expression of *RBR* in the sensitive genotypes could leads to the arrest of the CLAVATA-WUSCHEL feedback loop, resulting in cell differentiation and cell-cycle arrest reminiscent with the phenotype of the *wus* mutants.

Whether the expression changes of these genes of the above discussed cell cycle related gene are a secondary effect or the primary cause of the blindness phenotype is difficult to determine, although it is more likely that that they are indirectly affected in the blind seedlings, because neither *CYC nor RBR* genes were present in the list of differentially expressed genes located in the QTL region. Though, an interesting candidate gene involved in the cell cycle was present in this list, which

is a member of the Mini Chromosome Maintenance (MCM)2-3-5 protein family. These proteins, first discovered in yeast but present in all eukaryotic genomes, form a helicase complex that has a role in both the initiation and the elongation phases of eukaryotic DNA replication (Tye 1994). Consistent with a role in DNA replication, *MCM* genes from Arabidopsis (Springer, McCombie *et al.* 1995) are preferentially expressed in young tissues that contain a high number of replicating cells, like embryos, young organs and meristems. Knock-out mutants of the *PROLIFERA (PRL)* gene are embryo-lethal demonstrating its essential role in cell division and tissue growth. It is conceivable to hypothesise that the down-regulation of the *MCM2-3-5* homolog in sensitive brassica plants underlies the arrest of cell division and hence loss of meristematic activity in the SAM.

A possible trigger for the molecular and subsequent cellular and morphological effects could be that imbibition at low temperatures leads to DNA damage. Previously, it has been shown that sowing dry seeds at low temperatures can lead to imbibitional damage (Bewley JD 2013). As most other orthodox seeds, brassica seeds enter a quiescent stage at the end of seed-maturation. After water uptake the embryo has to reestablish its active state and commences cell division and growth. These processes are well organized in time, for instance, DNA repair precedes DNA replication (Van Pijlen, Groot *et al.* 1996). It is known that DNA damage is signalled as stress and as a result the cyclin-dependent kinase (CDKA)-cyclin(CYCD) complex is kept in a phosphorylated and therefore inactive state, in which it fails to phosphorylate RBR and the cell is prohibited in entering the S-Phase of the cell cycle (Nasmyth 1995; Novak, Csikasz-Nagy *et al.* 1998). However, the general mitotic signal is present and therefore cyclin genes are expressed at first but in an uncoordinated manner, in a similar way as observed in this study.

Conclusion

Cold imbibition in sensitive brassica seed-lots induces SAM arrest. In addition to this inductive treatment, a genetic factor is partly responsible for the occurrence of blindness in brassica, which was confirmed by the discovery of a QTL. 39 differentially expressed candidate genes were identified within this QTL region. These genes showed no response in their expression levels due to cold, indicating that they are related to the sensitivity to blindness independent of the environmental conditions. Eight selected genes with a fold change of > 1.4 obtained from the time-point two dataset, were all specifically expressed in the meristem, the embryo or the seed showing that our tissue sampling is indeed enriched for the meristematic region and that samples were taken at an early time-point soon after germination. Among the eight genes is a homolog of the *MCM2-3-5* genes known to be involved in DNA replication, which is particularly essential in fast dividing cells. Other differentially expressed genes involved in the cell cycle, but not located within the QTL region, are most likely indirectly affected in the sensitive genotype. Seedlings with arrested meristems have a defect in cell-cycle and DNA replication in nuclei of the SAM, which supports the gene discovery described above. Further studies are needed to unravel the genetic differences and the downstream processes affected in the cold-induced blindness in sensitive genotypes.

Material and Methods

Induction of blindness in brassica

To induce blindness the dried seeds were subjected to different periods of cold. The cold was applied as an imbibition treatment of seeds. The seeds were placed on filter paper wetted with Millipore water in Petri dishes placed in a Styrofoam box filled with ice. The box with seeds was placed for ten days in a closed incubator at 4°C in the dark. The temperature of the imbibing seeds during this period was around 1.5°C. Afterwards the seeds were sown in coco-peat in a growth chambers at 20°C with 8 hours light. Plants were scored by eye for the absence of the SAM after growing for two and a half weeks. Different temperature regimes were applied and actual temperature was measured with data loggers close to the seeds.

Root length measurement

Root length of developing blind and normal brassica seedlings were measured of 200 cold imbibed seeds of a sensitive line from the AGDH population. Seeds were equally distributed on 40 squared plates containing growth medium; Daishin agar (Duchefa Biochemie) with Murashige and Skook medium Gamborg B5 Vitamins (Duchefa Biochemie). Plates were placed vertically at 20°C for ten days to let the root grow in a vertical manner. The plates with the developing seedlings were scanned from day three onwards for four consecutive days using the Whinryzo program (Regent Instruments Inc. WinRHIZO Arabidopsis 2009c). Afterwards the primary root length was determined using ImageJ 1.44n software (http://rsb.info.nih.gov/ij/). Finally, after growing for ten days the seedlings were scored for blindness and correlation analysis between the occurrence of blindness and the root length was performed.

Gene expression analysis

Homologs of *A. thaliana* genes active in the SAM were identified in *B. oleracea* by BLAST using the BRAD website (http://brassicadb.org/brad/). Alignments of the best hits from the blast search were made using ClustalW program. After the identification of the homologous genes with highest homology scores in *B. oleracea*, primers were designed BoSTM (Forward: CCAAAATCATGGCTCATCCTCACTACC, Reverse: GACGAGCATGTTTCTTCCAGCCTC) BoYLS8 (Forward: CGGCCATGATTGGGATGAGACTTG, Reverse: GCTCGTACATGGTGTTGAAGTCTGGAAC). To measure gene expression of meristem marker genes in blind brassica seedlings, meristem enriched tissue of ten plants per biological replicate were dissected daily under a microscope at the desired time-point in triplicate. Total RNA was isolated from this material using the total RNA extraction kit from Invitrap® Spin Plant RNA Mini Kit (Stratec Molecular - Invitec). Afterwards, cDNA was synthetized from 1µg of RNA using the TaqMan reverse transcriptase kit (Roche). Finally, gene expression was measured with the primer combinations listed in table A, by Q-RT-PCR using SYBRgreen flurophor (Eurogentec). For gene expression analysis the Livak ($2^{-\delta\delta CT}$) was used.

Technovit embedding

For the microtome sections the material was fixed in 4% paraformaldehyde. Afterwards, the samples were transferred to 30% ethanol overnight. The next day, they were dehydrated until 100% of ethanol in steps of 30 minutes. Subsequently, the Technovit protocol was followed according to the manufacturer (Heraeus Kulzer). The Technovit blocks were cut with a microtome in sections of 3µm and toluidine blue was used for staining. Pictures were made with the Zeiss Axioskop epifluorescence microscope.

QTL mapping

For the mapping, seeds of the AGDH population (Sebastian, Howell *et al.* 2000) was induced according to the cold induction assay in a randomized block design. Afterwards seeds were sown in randomized blocks in coco-peat and placed in the green house set at 20 °C for 21 days, followed by scoring the number of blind plants by eye. Phenotype data were analysed with MapQTL6 (Churchill and Doerge 1994). MQM mapping and interval mapping were carried out with the same significant QTL as a result.

Confocal microscopy / EdU staining

For imaging nuclei with DNA replication activity, seeds were taken from the cold induction assay and directly placed on sterile growth medium (the same as for the root length measurements) containing 10µM EdU (Invitrogen) and germinated in a growth cabinet under 24 hours light and at 17°C. After 48 hours treatment time, apices were dissected and dehydrated and stained as described in (Schiessl, Kausika *et al.* 2012). Afterwards, the apices were imaged with a confocal laser scanning microscope (Leica SPE DM5500 upright microscope) using Leica AF 1.8.2 software (http://leica-microsystems.com).

Transcriptome analysis

RNA-isolation, library preparation and sequencing:

Total RNA was isolated as described above. Sequencing library were prepared with the Illumina library preparation kit according to the manufacturer manual.

Sequencing was done on the Illiumina Hi-Seq 2000 machine, paired end with 100bp read length.

Read mapping

RNA-seq reads were mapped against the genome of *Brassica rapa* version 197 (www.phytozome.org) using TopHat version 2.0.5 [1]. In addition to providing the *B. rapa* annotation version 197 (phytozome), the following settings of TopHat were modified: --min-intron-length 50; –max-intron-length 11000; -g 1; -M -N 10 –read-edit-dist 10 –read-gap-length 4 –no-discordant.

Transcriptome assembly

Cufflinks version 2.0.2 [2] was used for performing reference based transcriptome assemblies for each RNA-seq samples separately. The following parameters of Cufflinks were modified: --min-intron-length 50 --max-intron-length 11000 --max-frag-multihits 1 -u. The individual assemblies were merged into a final assembly using the Cuffmerge program provided with Cufflinks.

Differential expressed genes

Differentially expressed genes (merged assembly) were detected using the Cuffdiff (-u) program provided with Cufflinks at an FDR of 0.05.

Supplement

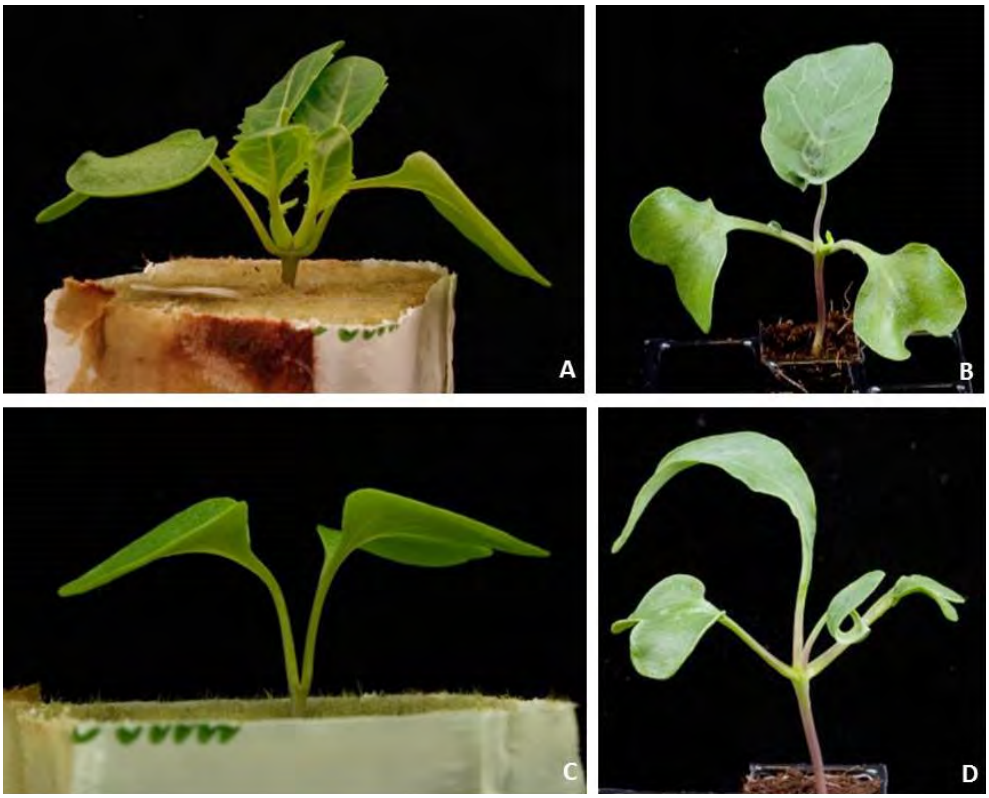

Figure S1. Blind brassica plants with: A) side shoot formation from the axils of the cotyledons, B) without leaves and C) with two aberrant leaves and D) with one aberrant leaf.

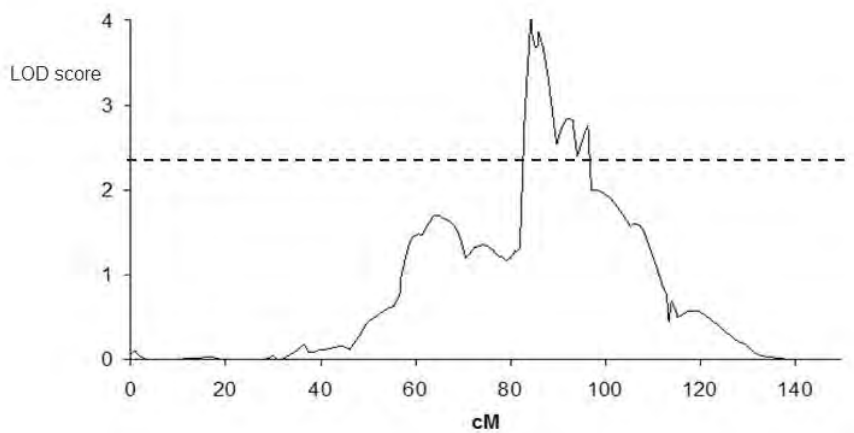

Figure S2. LOD score of the QTL on linkage group three. Dotted line represents the threshold given by permutation testing.

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A Seed Treatment to Preventing Shoot Apical Meristem Arrest in *Brassica oleraceae*

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Steven P.C. Groot

Chapter 3

Abstract

The shoot apical meristem (Yanai, Shani *et al.* 2005) of a plant gives rise to the aerial organs of a plant. Brassica (*Brassica oleracea)* plants can suffer from SAM arrest. These arrested plants are not able to form new leaves or reproductive organs, which often occurs directly after germination at the early seedling stage. We developed a seed treatment that prevents this SAM arrest in kohlrabi and cabbage seedlings. The method consists of soaking the seeds in deionized water at room temperature, followed by drying under mild conditions. Treated and untreated brassica seeds were compared with regards to their susceptibility to SAM arrest after exposing them to blindness-inducing low temperature conditions before sowing. Temporary controlled elevation of the seed moisture content gave a strong reduction in percentage of blind plants when compared to untreated seeds. This blindness inhibiting effect could only be obtained when seeds were dried back from a pre-soaking treatment at elevated seed moisture content of around 20%. In contrast, when temporary incubated at a higher seed moisture content the plants still suffer from SAM arrest, indicating that at higher moisture contents processes take place that keep or make the seed more vulnerable to SAM arrest. A time course experiment revealed that keeping the seeds at 45% seed moisture content increases the sensitivity to blindness over time. Temperature and time of soaking also significantly affected the effectiveness of the seed treatment. Our data revealed that by using a pre-soaking treatment it is possible to prevent blindness in susceptible brassica seeds and that the seeds show a developmental window in which it is more susceptible to external stimuli leading to SAM arrest.

Introduction

Brassica (*Brassica oleracea*) plants can suffer from shoot apical meristem arrest or 'blindness' (Wurr, Hambidge *et al.* 1996) (Chapter 2). The phenomenon occurs in kohlrabi, broccoli as well as in other brassica crops and can lead to high economic losses for growers. This blindness syndrome can manifested by a variety of abnormal phenotypes, including seedlings without apical shoot and leaves or seedlings with one, two or very few true leaves which are often distorted and severely reduced (Chapter 2).

The physiological basis of blindness is still not well understood. Previous studies on SAM arrest in brassica suggested that the exposure to low temperatures during plant growth is one of the main factors causing SAM arrest (Mounsey-Wood 1957). Recently, a method for inducing blindness in seedlings from sensitive brassica seed lots has been developed, which is also based on the response to cold (Chapter 2). This method allows predicting the susceptibility to SAM arrest of brassica seed batches. In this blindness screening procedure dry brassica seeds are imbibed on top of wet filter papers and exposed to 0-2°C for ten days to induce SAM arrest. In the same study, it was shown that blindness is significantly and proportionally influenced by the temperature used during this so-called "cold-induction", in which lower temperatures induced a higher number of blind plants.

Considering the previous studies on SAM arrest, it seems that cold water imbibition may lead to blindness either by causing direct damage to the meristem or by inducing a change in its physiological state. Whatever may be the origin of this syndrome, it remains meaningful to further investigate the effects of temperature during initial seed water uptake as such knowledge may reveal strategies for preventing blindness in brassica. In this study, we discovered that a pre-soaking of sensitive brassica seeds at room temperature, before the cold induction, had a preventive effect on the occurrence of blindness. This effect could be preserved even after drying the seeds back to the initial moisture content of 7% (on fresh weight basis), providing a potential method to prevent the initiation of blindness with susceptible seed lots from *Brassica oleracea* varieties. In this chapter we studied this preventive method in detail.

Results

Effect of water temperature during seed imbibition

Kohlrabi seeds develop arrested SAMs when the seeds are incubated for several days at low temperatures (0-2°C). In the standard blindness screening assay, Petri dishes are immediately covered with ice after seeds have been placed inside the dishes. The immediate exposure to 0-2°C results in a direct uptake of water by the seeds at nearly freezing temperature. To develop a method to prevent kohlrabi losing their meristem as a response to low temperatures, we studied the effects of seed water imbibition (pre-soaking) at room temperature (RT) before exposing the seeds to 0-2°C for 7 days and found that pre-soaking kohlrabi seeds with deionized water for 2 h at RT prior to cold imbibition significantly reduced the percentage of true blind plants from 17.9% (SD=1.6%) to 0.6% (SD= 0.8%). This indicates that blindness induction occurs when dry seeds absorb cold water and it can be prevented by allowing the seeds to absorb water at RT before being exposed to cold.

Seed treatment to inhibit shoot apical meristem arrest in Kohlrabi

As pre-soaking Kohlrabi seeds for 2 h at RT prevented to a large extent the formation of blindness, we were interested to implement this method in commercial seed handling, which requires drying of the seeds. Therefore, we tested if the preventive effect of the treatment would also persist after drying the seeds back to 6-7% moisture. We soaked and dried Kohlrabi seeds prior to the cold imbibition and measured the total number of blind plants after three weeks of outgrowth. The results of this experiment (Figure 1) were in agreement with our previous finding, confirming that pre-soaking the seeds prevented the formation of blind plants. We also found that drying the seed after pre-soaking did not abolish the effect of the treatment (Figure S1).

To analyze if the effect of the pre-soaking is dependent on the seed-batch or the genotype used in all previous described experiments, we treated another seed batch from ‘Opimes 273.580’ along with a seed batch from a different genotype (‘Olivia 188.902’). Figure 2 shows the results of the preventive treatment as compared to control after blindness induction. In both varieties, the total number of blind plants

was reduced more than nine-fold by the pre-soaking treatment as compared to untreated seed, with treated seeds reaching values of around 4% blindness or less. These results confirm that the preventive method is effective for different sensitive seed-batches of the same genotype as well as for different kohlrabi genotypes. Additionally, we tested the preventive treatment for a seed batch of a white cabbage variety ('Stanton'),that was sensitive to SAM arrest. Also for this genotypes the pre-soaking treatment reduced the number of blind plants substantially (Figure S2).

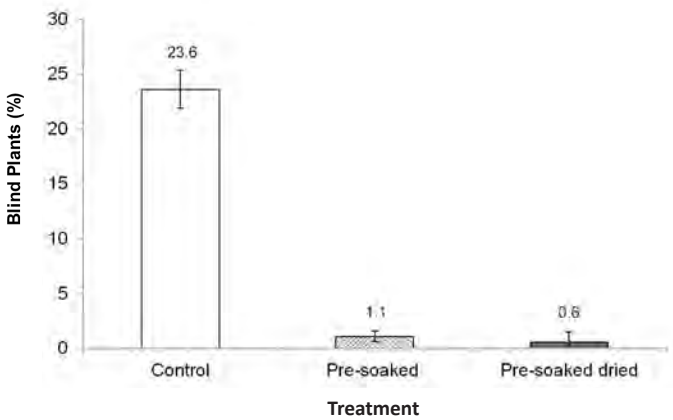

Figure 1.
Frequency of blind seedlings as affected by pre-soaking seed treatments for variety 'Opimes 273.586'. Control: untreated seeds; pre-soaked: seeds were allowed to uptake deionized water at 20 °C for 2 h; pre-soaked/dried: same as pre-soaked, but seeds were dried to 6-7% MC after water uptake before cold-induction at 0°C. Error bars represent Standard Deviation of four replicates with 120 seedlings.

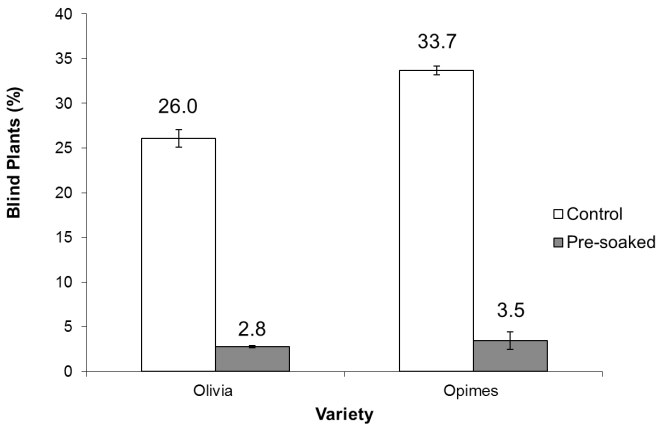

Figure 2.
Frequency of blind seedlings obtained by blindness preventive treatment in 'Opimes 273.580' and 'Olivia 188.902' (blindness screening test). Control: untreated seed; Pre-soaked: seed soaked in deionized water at RT for 2 h and dried to 6-7% MC. Error bars represent Standard Deviation of two replicates with 150 seedlings.

Optimization of the seed treatment against blindness

In the previous experiments, pre-soaking of kohlrabi seeds as a preventive treatment to inhibit the development of blindness was carried out at RT. We have observed that temperature has an effect on the SAM of kohlrabi seeds. Therefore, we analyzed the effect of the pre-soaking water temperature on the resistance of kohlrabi seeds to subsequent cold (Figure 3). We found that water temperature during pre-soaking significantly influenced the effectiveness of the preventive treatment. Increasing the water temperature from 16°C to 22°C reduced blindness from 12.7% to 5.7%, whereas a temperature increase from 22°C to 28°C reduced blindness by only an additional 1% , the difference between latter treatments being statistically non-significant (p>0.10, one-way ANOVA).

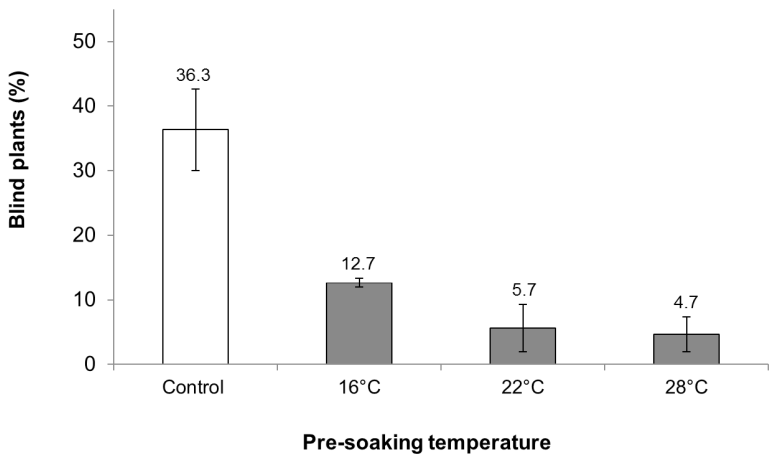

Figure 3. Effect of the temperature of pre-soaking on the frequency of blind seedlings in 'Opimes 273.580' (after blindness screening test). Control: untreated seed; '16 °C', '22 °C' and '28 °C': seeds pre-soaked in deionized water for 2 h at the corresponding temperatures and re-dried to 6-7% MC. Error bars represent Standard Error of two replicates with 150 seedlings.

Besides the water temperature we also tested if the pre-soaking duration has an effect on the Kohlrabi seeds (Figure 4). Increasing pre-soaking time at RT significantly reduced blindness in kohlrabi. Because exposure to extended periods of steeping may induce germination-related processes and compromise subsequent storability of the seed, and given the fact of the marginal increases on effectiveness, we assumed that pre-soaking for 2 h at RT, which already reaches a significant reduction on blindness, is possibly the optimal treatment for kohlrabi seed in practice.

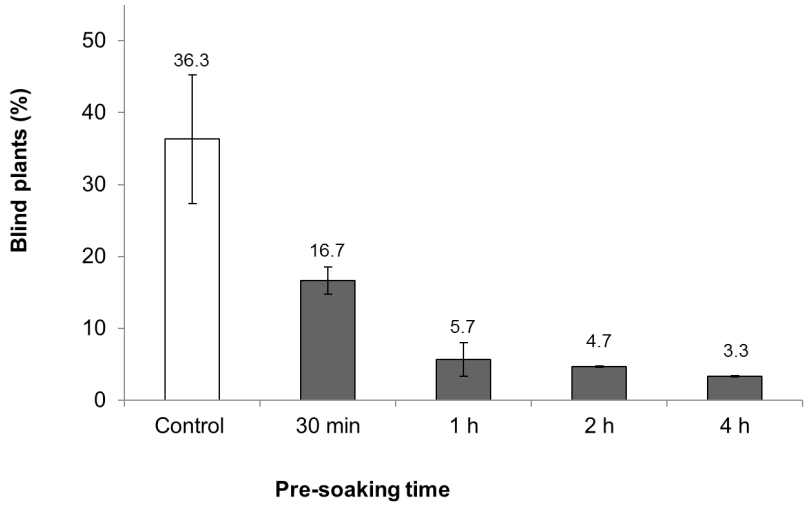

Figure 4. Effect of pre-soaking time on the frequency of blind plants in 'Opimes 273.580' (blindness screening test). Control: untreated seed; '30 min', '1 h', '2 h' and '4 h': seed pre-soaked in deionized water for the mentioned duration and subsequently dried to 6-7% MC. Error bars represent Standard Error of two replicates of 150 seedlings.

Elevating the seed moisture content increases the tolerance of seeds to the cold treatment

In the previous experiments, we found that two hours of pre-soaking at RT reduces substantially the development of blind plants after the cold treatment. In these two hours of imbibition seeds take up water and increase their moisture content. We wanted to know if certain moisture content is needed at which the seed increase their tolerance to the cold. We tested a range of moisture contents and found that elevating the seed moisture content to 20% (wet basis) already reduced the number of blind plants substantially (Figure 5). A further increase in moisture content did not reduce the development of blind plants significantly, indicating that pre-soaking to 20% seed moisture content is enough to increase the tolerance of the seeds to cold. The positive effect of the temporary wetting of the seeds could only be persevered in dried seeds when the seeds had reached a MC of 20 or 70% during the pre-soaking (Figure 5). Temporary wetting to 20% MC gave the same sensitivity as for 70%, whereas intermediate seed moisture levels resulted in higher frequencies of blind plants with a maximum of 60% after wetting to 45% seed MC. This frequency of blind seedlings is even slightly higher than that obtained from non-treated seeds.

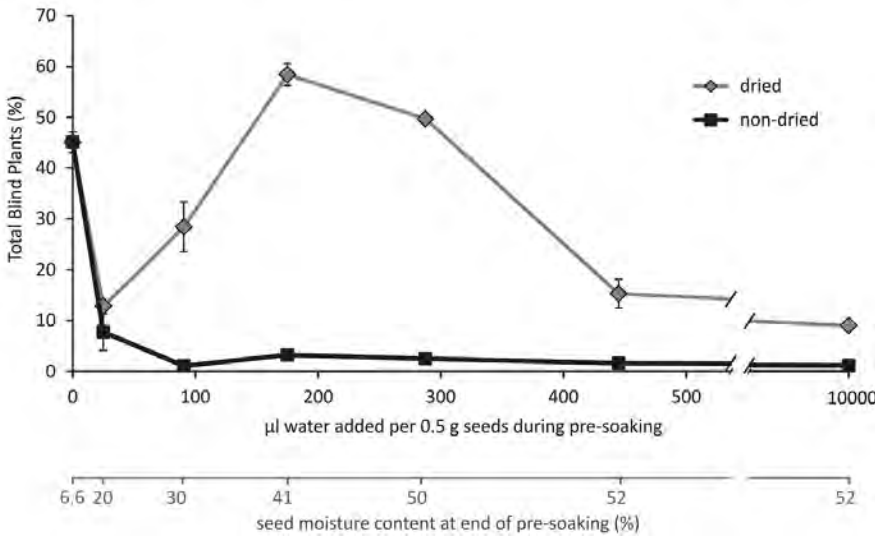

Figure 5. Effect of pre-soaking seeds for four hours at different moisture levels on subsequent sensitivity to the induction of blindness. After the pre-soaking seeds were subjected either directly to the cold induction assay (not dried) or after drying (dried). Error bars represent the standard error of two replicates of 0.5 g seeds.

We increased the seed moisture content gradually by prolonged incubation of the seeds under water limiting conditions, to a maximum of 45%. By this approach the MC increases and reaches 45% and is kept at that level (Figure 6). After different presoaking periods, the seeds were dried back and tested for blindness sensitivity. The lowest number of susceptible Stanton seeds was obtained after 1 hour pre-soaking, when the MC is approximately 35%. However, a seed moisture content of 45% is only reached after more than two hours of imbibition. With increasing time, incubated at moisture content 45% the seeds seem to regain their susceptibility to the blindness inducing conditions, reaching a maximum after about 8 hours.

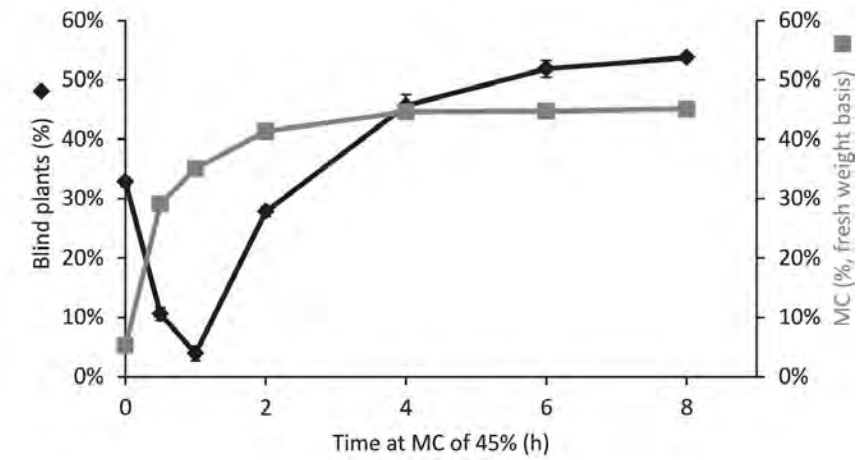

Figure 6. Effect of pre-soaking seeds for certain amount of time under water limiting conditions, followed by drying. Since water-uptake takes some time, the actual seed moisture level was determined at the end of the pre-soaking treatment (black line). Error bars represent the standard error of two replicates of 0.5 g seeds (grey line, % blind plants).

Discussion

Some seed lots from *Brassica oleracea* suffer from SAM arrest when the seedlings germinate under low temperature conditions (chapter 2). The susceptibility of a seed lot to SAM arrest can be tested via a screening method where seeds are imbibed at low temperatures before transferring them to standard growth conditions (Chapter 2). The screening method implicates imbibition of seeds at 0-2 °C for seven or ten days. After this cold imbibition, susceptible seeds develop into seedlings with a high percentage of arrested SAM.

The exact cause for meristem arrest in sensitive seed lots is not known, but it is clear that cold imbibition and subsequent incubation at low temperature is a key factor. In dry seeds the cytoplasm is in a glassy state and the membranes are in a dry bilayer (gel) state, which is poor barrier to leakage of metabolites and salts (Bewley 1997). Hydration of membranes during imbibition revert membranes from a gel phase to a liquid crystalline phase. But when imbibition takes place at low temperatures this phase transition is slow and cellular content may leak through the membranes, a phenomenon that is called imbibitional chilling injury (Bewley JD 2013). Partial

hydration of the seeds (by water vapour or limited water supply) and incubation at a higher temperature switches the membranes to the liquid crystalline state and when seeds are imbibed at such condition, there will be no or far less leakage and imbibitional damage (Bewley JD 2013). It might be that such imbibitional damage is directly or indirectly related to the meristem arrest with sensitive seed lots. However, this cannot be the only factor, since the frequency of arrested seedlings is increased with the incubation time at 0 °C (Chapter 2), whereas the imbibition per se is restricted to the first day. Low temperatures can also limit the activity of repair enzymes and may restrict the metabolic activity needed to provide energy for such repair.

DNA-damage in seeds occurs during seed drying and storage. Under optimal imbibition conditions this damage is repaired prior to DNA-replication and radicle protrusion, but DNA-repair could be inhibited by cold leading to seedlings with arrested SAM. Alternatively, the rapid water uptake of seeds during the first phase of germination may cause oxidative stress, that might lead to additional DNA-damage (Dandoy, Schnys *et al.* 1987).

In this study, we demonstrated that pre-soaking blindness-susceptible kohlrabi and cabbage seeds in water of RT temperatures prior to the cold imbibition for two hours, can effectively prevent cold-induced blindness. This pre-soaking effect on reducing the sensitivity of the seeds to blindness induction is affected by the period of pre-soaking, the moisture content during the soaking and the drying after the soaking. This shows that not only moisture content is important but also the time that the seeds stay at that moisture content. Since temporary soaking changes the sensitivity of the seeds to SAM arrest upon subsequent cold imbibition, it is likely that some physiological or physical changes occur in the seed during the soaking period.

The optimum treatment period to reduce cold induced blindness is one (‘Stanton’, Figure 6) maximal two (‘Opimes’, Figure 4) hours of pre-soaking, indicating that upon imbibition at room temperature these changes are induced in a relatively short period of time. The experiments with water limitation during the imbibition at RT (Figure 4), showed that the changes can occur also at relative low seed MC.

Seeds generally absorb water more slowly at lower temperatures (Allerup 1958), nevertheless, increasing the water temperature to 28°C does not further reduce the amount of blind seedlings. It is possible, that at room temperature is the optimum temperature for these seeds or the rate of water uptake of the seed is not the critical factor.

As most other orthodox seeds, brassica seeds enter a quiescent stage at the end of maturation. After water uptake the embryo has to reestablish its active state and commences cell division and growth. These processes are well organized in time, for instance DNA repair precedes DNA replication. The processes that initiate upon imbibition, are also affected by the water activity or moisture content of the seeds (van Pijlen, Groot *et al.* 1996).

We have shown that both soaking time and seed moisture level influences the number of blind seedlings. Moreover, the effect of pre-soaking on the development of blind seedlings can only be preserved in dried seeds after soaking the seed to a moisture content of 20%. At 20% MC, the water activity in the brassica seeds is around 0.95, enough to initiate metabolic activity and germination related processes, while at 10% MC the seeds have a water activity of only about 0.6, which is too low for respiration (Vertucci 1989) and activity of most enzymes (Labuza 1971).

Arabidopsis thaliana seeds show enhanced transcriptional activity already one hour after start of the imbibition and the genes that are differentially expressed are mainly stress response genes (Preston, Tatematsu *et al.* 2009). Repair processes, for instance for DNA damage, occur early during seed imbibition (Bewley JD 2013) and at relatively low water levels. Relief from the sensitivity to SAM arrest was already observed in brassica seeds imbibed at 20 °C for one or two hours. It might be that this period, plus drying time, was enough to induce the production or activity of repair enzymes.

Priming, for instance through aerated hydration treatments, can improve seed vigor and the response of seeds to stress during germination (Mehra, Tripathi *et al.* 2003)(Powell, Yule *et al.* 2000). Low vigor seed lots with an accumulation of DNA damage in the dry seed can also contribute to an increase in the number of

abnormal seedlings produced (Thornton, Collins *et al.* 1993). In low vigour Brussel-sprout seeds the accumulated DNA damage is repaired during eight hours aerated hydration, before the onset of DNA replication, which might be the reason for an increase in the number of normal seedlings after aerated hydration of a low vigour seed lot. Interestingly, seeds that are primed at relative low moisture levels, e.g. a few days at 100% relative humidity, which does not permit DNA replication, are more tolerant to storage stress (Van Pijlen, Groot *et al.* 1996). This might render the seeds sensitivity to low temperature incubation at 0°C, resulting in more normal seedlings.

It is possible that the mechanism behind the reduction of the sensitivity to cold-induced blindness could involve DNA repair. Possibly, in the short imbibition time at room temperature the seeds are able to repair their DNA damage, which is induced during drying and storage, and hence, they can cope better with the subsequent cold. Alternatively, it might be glass phase transitions in the cytoplasm due to the temporary hydration (Bewley JD 2013), result in reshuffling of molecules reducing the sensitivity of the seeds.

Our data show that the susceptibility of the seed changes with the moisture contents. A schematic representation of the sensitivity shift with increasing seed moisture content can be seen in Figure 7. It remains unclear why a further increase of seed moisture content during the temporary hydration treatment re-establishes the sensitivity to the blindness inductive conditions after drying. DNA-repair could still take place at this moisture content and can therefore not be the reason for the cold-induced SAM arrest. During prolonged imbibition of the seeds, they enter into phase II of germination (Bewley JD 2013) and start preparing for cell division and growth. During this phase, part of the protection mechanisms is removed, e.g. LEA proteins (Soeda, Konings *et al.* 2005) and nuclei that are condensed during the quiescent phase regain their normal size(Van Zanten, Koini *et al.* 2011). In relation to that, primed seeds are generally more sensitive to storage under controlled deterioration test conditions (Van Pijlen, Groot *et al.* 1996). In our experiments this is also observed with the brassica seeds upon prolonged soaking treatments (Figure S1).

The results of the time course experiments (Figure 6) hint towards a process that either accumulates or degrades a substance or a transcript, as the percentage of SAM arrested seedlings increases with time. However, more research is needed to elucidate this process and to identify these molecules. A good strategy could be to analyze the metabolites and transcripts produced and degraded at 45% seed moisture content and compare it with data obtained with 20% seed MC. To date, no reports are available that show an effect of the imbibition conditions on shoot apical meristem functioning in seedlings, although, it is known that seed treatments like osmo-priming have a positive effect on stress tolerance and germination performance of seeds (Chen, Fessehaie *et al.* 2012)? . This demonstrates that during imbibition processes are activated that affect seedling vigor and stress tolerance during germination.

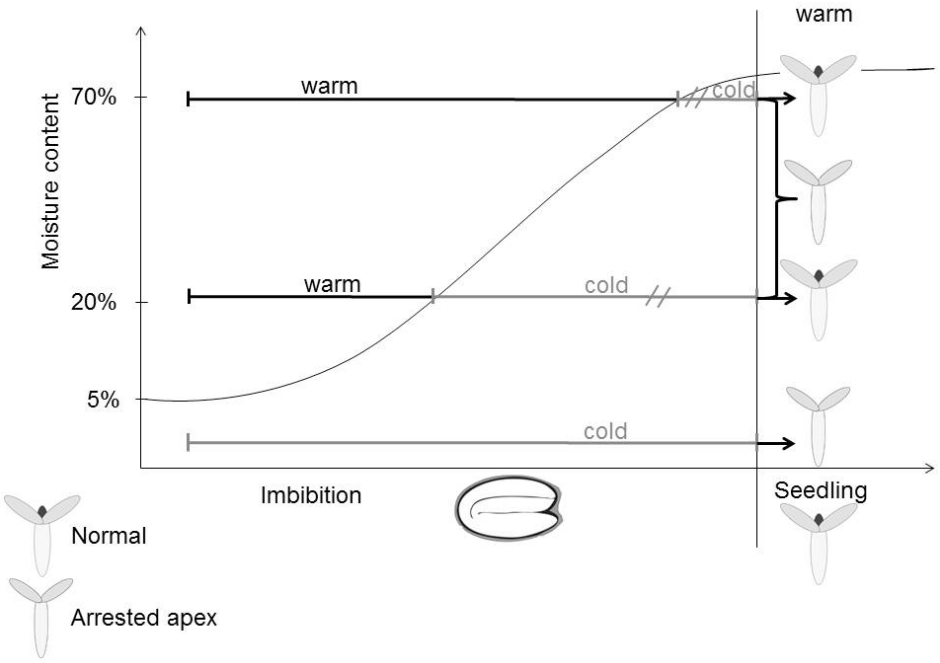

Figure 7. Schematic presentation of the relationship between imbibition temperature and the resulting phenotype after subsequent drying of the seeds to initial moisture content. Depending on the seed moisture content cold can induce the loss of the shoot apex , if the seeds are dried back to their initial moisture content of 5-7%. If cold is applied after the seeds reach a moisture content of 20% the seedling will develop a normal shoot. Applying the cold to seeds with a higher moisture content up to 70% will not rescue the shoot and the seedlings fail to develop true leaves. Arrows point to the resulting phenotype after a certain treatment. Warm means room temperature, cold mean 0-2˚C, which is applied always for the same duration (10 days).

Conclusions

We conclude that through a simple procedure as reported here, it is possible to prevent blindness in kohlrabi when it is caused by exposure to low temperatures. The method can be easily up-scaled for implementation at the commercial level. We discovered that the pre-soaking effect can be maintained after drying the seeds when the seed moisture content during soaking was either 20% or at least 70%, but that in-between another unknown mechanism takes place that re-induces the sensitivity during the pre-soaking or subsequent drying. This phenomenon is interesting to study in detail in the future.

Materials and Methods

Seed Samples

Seeds from kohlrabi varieties Opimes (batch no. 273.580 and 273.586, from 2009 harvest), Olivia (batch no. 188.902, from 2006 harvest) and the cabbage variety Stanton (batch no. 1645, from 2009 harvest) were used in this study. After harvest, seeds were stored at 15 C and 30-35% relative humidity (RH) until experiments were started.

Screening test for shoot apical meristem arrest

For blindness testing, we used the screening developed (chapter 2) with small modifications. For each individual experiment hundred seventy seeds were placed inside a 9 x 2 cm Petri dish containing two paper disks and 9.75 ml of deionized water. Petri dishes were side-wrapped with Parafilm, placed inside a polystyrene box and immediately covered with ice. The samples were maintained at 0-2°C for 7 days or 10 days in case of 'Stanton' seeds. After the cold incubation, the seeds were taken out from the Petri dishes and superficially dried using paper towels. Seeds were then directly sown on a wet peat block (4 x 4 cm) and covered by a 1-2 mm layer of wet sand. The trays were transferred to a growth chamber set at continuous 20°C and 16-h daylight with a light irradiance of 50 µmol. Trays were watered three times a week with tap water at 20°C. Three weeks after sowing, when normal seedlings

have reached two fully expanded leaves and showed a visible vegetative apex, the number of blind plants per tray was determined by visual scrutiny. Blind plants were sub-classified into three groups: (1) 'true blind seedlings', or seedlings with no apical shoot or a severely abnormal apical shoot, with either no leaves or with extremely small leaves (<3 cm in length); (2) 'one-leaf blind seedlings', seedlings with one true leaf and no or severely reduced apical shoot; and (3) 'two-leaves blind seedlings' seedlings with two true leaves and no or severely reduced apical shoot.

Effect of water temperature during seed imbibition

Seeds of 'Opimes 273.580' were placed inside Petri dishes as described in the screening method, but allowed to take up water at room temperature (pre-soaking) for 2 h before being transferred to 0°C. After the 7 days cold induction period, pre-soaked and control seeds were sown on trays containing 120-rock wool plugs, each plug being 2.4 cm in diameter. The plugs were kept moist by placing the trays inside plastic boxes containing tap water. The outgrowth assay was conducted in quadruplicate analysis inside growth chambers under continuous light (75 µmol light irradiance) and 20°C. 'True blind plants' (see above for description) were counted 3 weeks after sowing.

Development of a seed treatment against blindness

Treated and untreated seeds of 'Opimes 273.580' and 'Olivia 188.902' were tested following the blindness screening assay as described above. To standardize the soaking procedure and make it feasible for up-scaling, seeds were directly soaked in deionized water instead of being placed on top of wet filter papers. Three hundred and forty seeds (6-7% MC) were placed inside a 150 ml glass beaker containing 100 ml of deionized water. The seeds were soaked at room temperature (22-23°C) for two hours under constant stirring on a magnetic stirrer set at 400 rpm. Afterward, seeds were separated from the soaking water by vacuum filtration. In the 'pre-soaked' treatment, seeds were directly tested wet, while in the 'pre-soaked, dried' treatment, seeds were dried at 25°C and 30% RH after soaking using a climate test chamber until they reached 6-7% MC before being tested. The above-described method to prevent blindness was optimized by testing different temperatures

(16°C, 22°C and 28°C) of soaking water and times of soaking (30 min, 1 h, 2 h and 4 h) at room temperature (RT). Temperature treatments were done inside climate test chambers.

Limited moisture level treatment

Cabbage seeds of the variety "Stanton" were hydrated (pre-soaked) to a specific moisture contents (MC) by mixing the seeds with a specific amount of water for four hours, at room temperature and subsequently dried back to 6% seed MC. Alternatively, the seed MC was increased to 45% in the same way as described above and seeds were kept for either 1, 2, 4, 6, or 8h, followed by drying to 6% seed MC. Dry non-treated seeds, that were immediately exposed to the blindness screening test, served as control (see above).

Seed moisture content determination

Seed MC, on fresh weight basis, was gravimetrically determined in duplicate analysis of 500 mg seeds after drying the seeds in a forced air oven set at 130°C for 1 h.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed using GenStat software version 14 (Payne 2009) to make statistical comparisons among certain treatments.

Supplement

Storage experiments

Treated (2 h soaking with deionized water at RT and dried) and untreated seeds of 'Opimes 273.580' and 'Olivia 188.902' were stored in paper bags for 3, 8 and 12 weeks inside a test chamber set at constant 30°C and 76% RH, established by placing a container filled up with a NaCl saturated aqueous solution inside the chamber. After the mentioned storage periods, seed sub-samples were taken out of the bags and germinated on top of paper at 20°C/30°C (16 h and 8 h, respectively) and continuous light (ca. 75 µmol) following the guidelines of the International Seed Testing Association (Anonymous, 1993). Each germination test was done in quadruplicate analysis of fifty seeds.

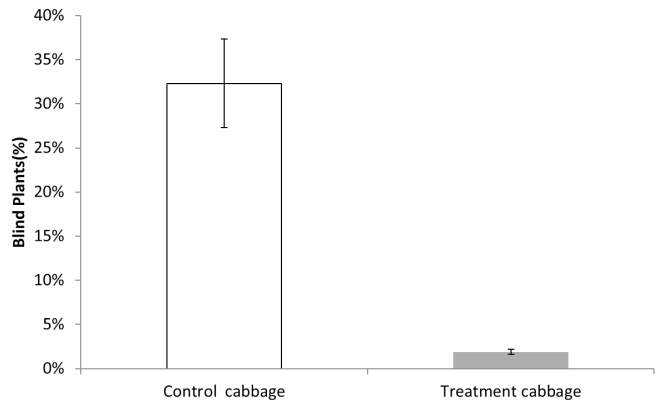

Figure S1:
Total number of blind seedlings affected by blindness preventive treatment in white cabbage variety "Stanton" Control, untreated seeds; 'treated', seeds soaked in deionized water at RT for 2 h Error bars represent standard error of three replicates of 300 seeds.

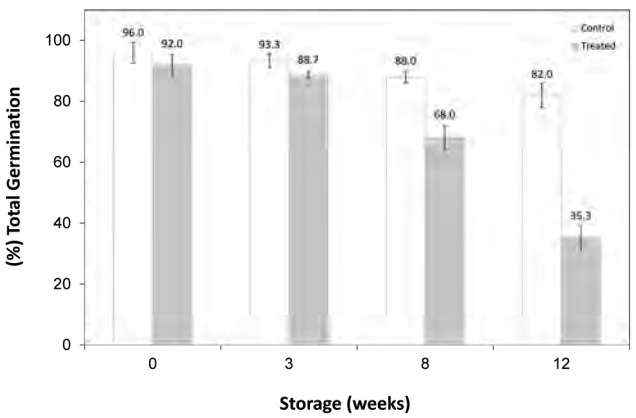

Figure S2.
Evolution of total germination percentage after 0, 3, 8 and 12 weeks of controlled deterioration with 'Olivia 188.902' 'Treated' seeds were pre-soaked for two hours. Error bars represent standard deviation of four replicates of 50 seeds.

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Abiotic stress induces shoot apical meristem arrest in Tomato (*Solanum Lycopersicum*)

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Chapter 4

Abstract

All areal parts of a plant derive from a group of undifferentiated cells in the shoot apical meristem (SAM) (Yanai, Shani *et al.* 2005). If these cells stop dividing further development is prohibited. Certain tomato (*Solanum Lycopersicum*) cultivars are suffering from sensitivity to this phenomenon. The SAM arrest can occur early during the young seedling stage or later in development. Not in all cases these arrested and so-called blind or bud-less-seedlings stop with further plant growth, some have the ability to recover their growth via the formation of side shoots. Only few studies have been conducted exploring the origin of this SAM arrest. In general it is believed that it is caused by particular unfavourable growth conditions, because the frequencies and phenotype of those aberrant plants vary between growing seasons. We studied the environmental conditions at which plants from sensitive tomatoseed lots lose their SAM and focussed on the effect of abiotic stress factors. We found that high temperatures, especially early during germination, induce SAM arrest in sensitive tomato seedlings. These conditions could only cause SAM arrest in a specific developmental window of the first three days during seed germination. This period is before radicle protrusion, indicating that the seed imbibition conditions have a direct effect on SAM development during the subsequent seedling phase. Analysis of different seed lots from the same varieties showed differences in the sensitivity of the seeds towards induction of blindness, demonstrating that seed production or treatment conditions can influence the sensitivity of the seeds. The work shown here paves the road for further detailed studies to unravel the underlying molecular mechanisms controlling SAM arrest in tomato.

Introduction

Apical meristems of plants can be divided into three different zones: the peripheral zone, the central zone and the ribzone. While the central zone acts as a reservoir of stem cells, the ribzone gives rise to the stem tissue and the peripheral zone produces the lateral organs (Steeves T.A. 1989; Meyerowitz 1997; Barton 1998). In indeterminate tomato (*Solanum Lycopersicum*) genotypes, the shoot apical meristem (Yanai, Shani *et al.* 2005) produces a certain number of leaves before transition to an inflorescence meristem and the axillary meristem of the last leaf develops into the new SAM. Some tomato seed-lots show a disturbance of leaf production and the SAM stops the initiation of new leaves, which can happen either at early or later stages during plant development. Tomato seedlings with arrested meristems are often called “blind” or “bud-less” by plant nurseries and are retarded or completely arrested in their growth. These blind plantlets are a serious problem not only for the nurseries, but also for tomato growers when this phenomenon occurs at a later stage of plant development (Wetzstein and Vavrina 2002) (Wetzstein and Vavrina 2002). It has been reported that the occurrence of blind tomato plants may vary in an unpredictable fashion from 10% to 90% in standard production conditions and therefore being a high risk for production-losses for the farmers (Wetzstein and Vavrina 2002). Furthermore, it has been suggested that there is genetic variation for SAM arrest in tomato, because some varieties are known to be more susceptible for the loss of the meristem activity than other genotypes (Buitelaar 1995). This indicates that both genetic and environmental factors influence the sensitivity to this phenomenon in tomato.

Shoot apical meristem arrest is also known in other crops including baby’s breath (*Gypsophila paniculata*) (Hicklenton, Newman *et al.* 1993), cauliflower and broccoli (both *Brassica oleracea*) (Forsyth, Barnett *et al.* 1999a). Previous studies on SAM arrest in brassicas suggested exposure to low temperatures during plant growth as one of the main factors causing SAM arrest (Mounsey-Wood 1957). It was shown that if calabrese was (*Brassica oleraceae* var. italica) cultivated during the winter period, blindness occurred and that it is characterized by a cessation of leaf primordium production by the vegetative apex (Forsyth, Barnett *et al.* 1999a).

In this chapter, we present that growth conditions during early tomato seedling development play an important role in the induction of SAM arrest. We studied the effect of temperature, light and priming on the occurrence of blindness in tomato and developed an assay to induce blindness at the seedling stage in sensitive seed lots. Furthermore, we were able to pinpoint the sensitivity window for the perception of the temperature signal to lose the apical meristem to the first three days of seed germination. In addition, our results suggest that a genetic component might be involved in blindness sensitivity because different genotypes respond differently with respect to blindness. When blind plants and normal plants from a genetically identical population of F1 hybrid plants were separately propagated and analysed in the F_2, both normal and blind plants appear in the two offsprings, indicating that the blind phenotype is not epigenetically inherited. Analysis of different seed lots from the same variety showed differences in the sensitivity of the seeds with respect to induction of blindness and it appeared that priming can enhance the sensitivity, showing that seed production and treatment conditions can influence the sensitivity of the seeds.

Results and Discussion

Morphology of blind tomato seedlings

During the first stages of tomato plant development seedlings can lose their shoot apical meristem. We analysed the phenotype of normal and arrested plants at a macroscopic level (Figure 1, Table 1) and observed that this arrest most often occurs after the initiation of two leaves. Figure 1A depicts a normal plant with a functional apex flanked by developing leaves. In comparison to a normal plant, plants with an arrested apex, often have only one (Figure 1B) or two leaves that are fused together at the stem. Each of the leaves in blind seedlings having only two leaflets (Figure 1C) whereas the first leaves in normal plants have three leaflets. Although the arrested seedling type shown in Figure 1B is the most frequently observed one, there is large phenotypic variation (Table 1 and Figure 1 in the supplement at the end of this chapter). This variation is mainly expressed in leaf number, in some cases the

tomato seedlings form no leaves at all, one leaf, or even up-to ten leaves before the SAM is impaired in producing new leaves. Furthermore, the few leaves formed can either be of normal architecture or aberrant (Figure 1B, Figure S1). Once arrested, plants may remain without a shoot and further leaf development from the original SEM. However, some plants are able to form new shoots from axillary meristems present in the axils of the cotyledons or the first leaves, although this leads to a large developmental delay (Figure S1). These side-shoots develop further normally and are able to flower and produce seeds. Next to growth arrested plants, we also observed seedlings containing a normal shoot without clear internodes between the first two leaves and the shoot is positioned abnormally in the middle of the two leaves (Figure S2). We will further refer to such plants as abnormal (Table 1).

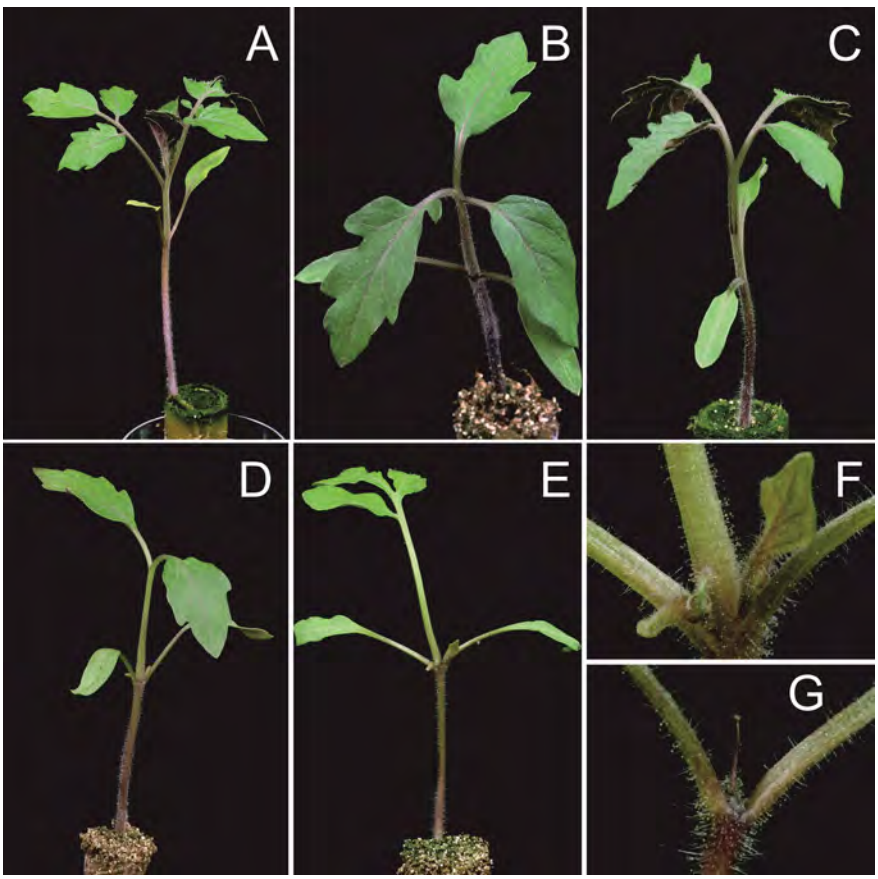

Figure 1. Phenotypic variation of three weeks old tomato plants (cv. Kinsberg): A: Plant with normal shoot and leaves. B-E: Plants with aberrant or arrested shoot and leaf growth. F: Close-up of the SAM region of the plant shown in picture E. G: Blind plant with a pistil-like structure between the cotyledons.

Table1. Classification of phenotypes: Different phenotypes observed with plants of cultivar 'Kinsberg'.

Phenotype	Category
Normal plant with apex and axillary buds	Normal
No leaves , no apex, and no axillary buds present	Blind
No leaves, no apex, axillary buds present with cotyledons	Blind
One leaf, no apex, and no axillary buds present	Blind
Two leaves, axillary buds present, but no apex	Blind
Three leaves, no apex, and no axillary buds present	Blind
Fused first two leaves, axillary buds present, but no apex	Blind
First internode missing, no apex, and no axillary buds present	Blind
First internode missing, axillary buds present, but no apex	Blind
First leaf missing, further normal plant	Abnormal
Fused first two leaves, further normal plant	Abnormal
First internode missing, further normal plant	Abnormal
Phenotype difficult to score, e.g. too small	Unknown
Non-germinated	Not germinated

Blindness observed in tomato varieties

Priming is a common seed-treatment for tomato seeds, used to promote or synchronise germination of a seed-batch and performed by controlled hydration of seeds followed by drying. Seed-batches showing high frequencies of blindness were reported by nurseries, especially with primed seeds. We obtained seeds from such commercially primed seed lots and corresponding non-primed seeds from the same seed lot. The non-primed seeds showed almost no blind seedlings, in contrast to the primed seeds (Figure 2). This finding indicates that priming of seeds is a potential cause of blindness. Due to the fact that commercial priming protocols of are secret, our own hydro-priming protocol was applied to a sample from the same non-primed seed-batch. Only a minor, but non-significant, increase in blindness was achieved with our priming method compared to the non-treated control and far less compared to the seeds primed according to the company's protocol (Figure

2). It is possible that the difference between both priming protocols is causing this discrepancy, or that another unknown factor, for example during drying of the seeds, causes an increase in the frequency of blind plants after the company priming.

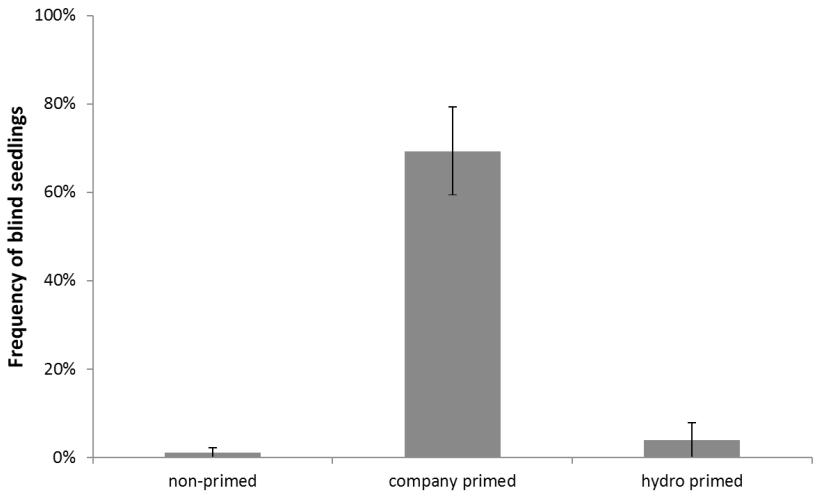

Figure 2. Frequency of blind seedlings produced from tomato seeds of the cultivar Variety 3, before priming and after priming with two different priming methods, either performed by the seed company, or by us through hydro-priming (6 days, 25°C, 39% final moisture). Error bars represent 95% confidence interval for 84 seedlings.

In addition to these results, another company reported high frequencies of blind seedlings after priming, although varying between seed lots. Seeds of this variety had been produced in three different countries on three different continents: The Netherland, Chile and Kenya. The non-primed seeds of this variety indeed developed almost no blind seedlings (Figure 3), while seeds primed by the company, showed a high frequency of blind plants, although only with one type of priming treatment. This time we were able to induce the development of blind seedlings (Figure 3), using our standard hydro-priming treatment. However, blindness induction via priming was only possible with the seed lot that was produced in the Netherlands. Seeds from the other seed-production locations developed no blind seedlings after the same hydro-priming treatment. These results indicate that seed-priming can induce blindness in tomato and that the sensitivity is dependent on the history of the seed lot. These first indications of seed-treatments and the seed batch

history affecting the induction of blindness in tomato encouraged us to perform further investigations. This was done on the variety 'Kinsberg', that was reported to produce high frequencies of blind seedlings without priming and from which enough seeds were available. This seed lot was used in the experiments for the phenotyping (Figure 1, Table 1) and for further studies.

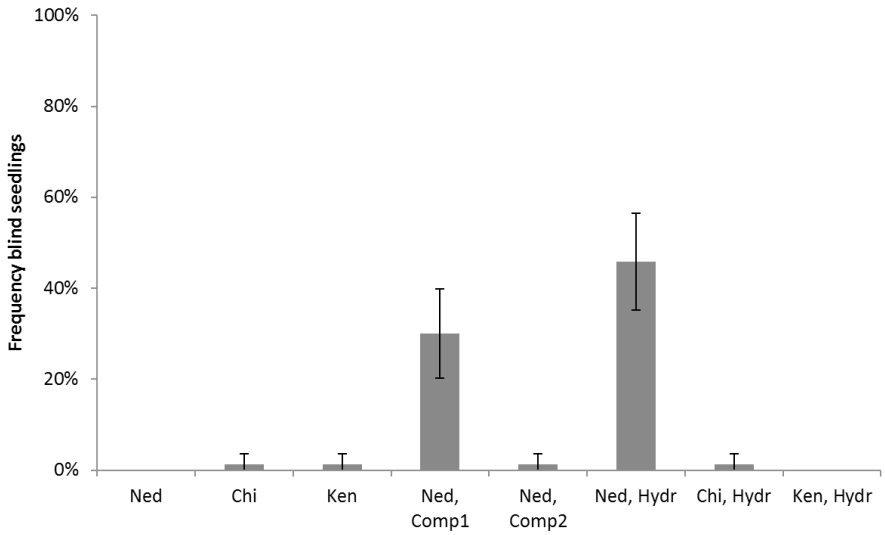

Figure 3. Frequency of blind seedlings with tomato seed lots (cultivar 'Oscar') produced in three different countries (Ned = the Netherlands, Chi = Chile, or Ken = Kenya). Seeds were either non-primed or primed by a seed company (two independent treatments: comp 1 and comp2) or hydro-primed (Hydr, 6 days, 25°C, 39% final moisture). Bars represent 95% confidence interval for 80 seedlings per sample.

4

High light intensities increase the number of blind tomato seedlings

Commercial tomato transplant production in greenhouses generally takes place with additional artificial illumination. Recently, it was demonstrated that tomato organogenesis is influenced by light, as tomato meristems cease leaf initiation when exposed to the dark (Yoshida *et al.*, 2011). To test whether light can influence SAM arrest, we produced seedlings from tomato cultivar 'Kinsberg' at different light intensity levels (50, 100, 150 and 600 µmol) (Figure 4). With increasing light intensities, the frequency of blind tomato plants increased significantly (p-value <0.001). It rose from 40% at 50 µmol light up to 70% at 600 µmol. Already an increase from 50 µmol to 100 µmol light resulted in a significant increase in blindness. This is surprising, because it is in contrast to the finding of Yoshida *et*

al. (2011), where they showed that leaf initiation ceases in the dark. To further study the role of light in blindness, we investigated whether the light-sum would influence the frequency of arrested plants. Therefore, we exposed the seedlings to a 16/8 hours light/dark regime or continuous light. No significant effect of the day length on the percentage of blind tomato seedlings was detected but the effect of increasing light intensity was affirmed.. In a recent study, it has been shown that inhibition of Arabidopsis seed germination in the absence of light by the Phytochrome Interacting Factor 3-Like5 (PIL5) transcription factor involves interaction with several hormone related transcription factors (Oh *et al.*, 2007).

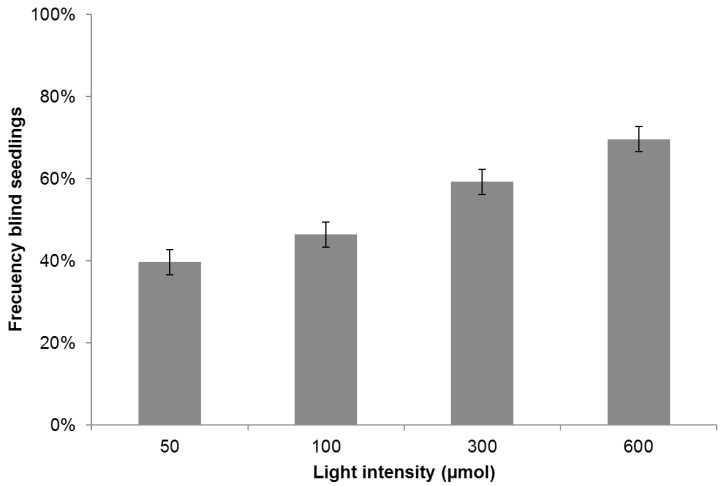

Figure 4. Frequency of blind of blind tomato seedlings (cv 'Kinsberg') after three weeks of growth at either 50, 100, 300 or 600µmol intensity light at 25 °C and a day-length of 16h. Error bars represent the standard error of two replicates of 328 seeds. Letters (a,b,c) represent stistical different subsets (Student's t-test, P=0.05). Averages, SE and t-test were calculated after probit.

Plant hormones play an important role in seed germination. Gibberellins, ethylene, brassinosteroids, and cytokinins promote germination, while abscisic acid and jasmonic acid inhibit this process (Davies and Finkelstein, 2010). These hormones are involved in the signal transduction pathways of environmental signals, such as water content, light and temperature, to make sure that the seed only germinates under favourable environmental conditions (Finch-Savage and Leubner-Metzger, 2006). Hence, it is possible that light is a signal for SAM arrest in tomato, because it is perceived already in imbibing seeds. However, it is not clear how this stress can lead to the arrest of the meristem. It is known that the Arabidopsis homeobox gene

WUSCHEL (WUS) specifies the stem cell fate in the meristem and that the expression level of the tomato ortholog is not affected by light in tomato SAM (Yoshida *et al.*, 2011), suggesting that the light signal does not influence directly the key component *WUS*.

High germination temperature increases the number of blind tomato seedlings

Despite that the seedlings were produced in a temperature controlled climate room, it could not be ruled out that the effect of high light intensity on blindness induction is at least partly due to a local temperature increase caused by the irradiation. Indeed, analysis of the temperature in a climate room set at 25 °C with 600 µmol light revealed that at the position of the imbibed seeds a temperature of 29 °C was reached, whereas the temperature at that the same position was only 24 °C in the treatment with 100 µmol light: a difference of 5 °C. The slightly lower temperature under low light condition, is likely due to evaporation of the wet rock-wool blocks.

To study if the seeds or seedlings are sensitive to a relative high temperature we tested if the effect of light intensities (100 or 600 µmol light) on blindness induction is consistent at different temperature regimes (20 °C or 25°C) (Figure 5). In both temperature regimes the plants responded to higher light intensities with an increase in SAM arrested plants. Additionally, we found that with the environment set at 20 °C and 600 µmol light the percentage of blind plants was only a third of the amount when exposed to 600 µmol light and set at 25ºC (Figure 5). The seedlings produced at 20 °C and 100 µmol light exhibited only 4% blind plants. These results point towards temperature as the main or even only factor in stimulating blindness in tomato seedlings under these conditions. This observation confirms other reports that temperature stress can induces SAM loss in tomato (Mounsey-Wood 1957) and (Forsyth, Barnett *et al.* 1999a). The effect of higher light intensities on the occurrence of blindness in tomato seems to be mediated through a temperature increase at the seed or seedling level.

The optimal temperature for tomato seed germination, estimated by rate of radicle protrusion, is between 25 and 30 °C (Labouriau and Osborn 1984). These are also the temperatures often used in tomato transplant production. Based on our observations, it is better to use a lower germination temperature for seeds that are sensitive to the induction of blind plants.

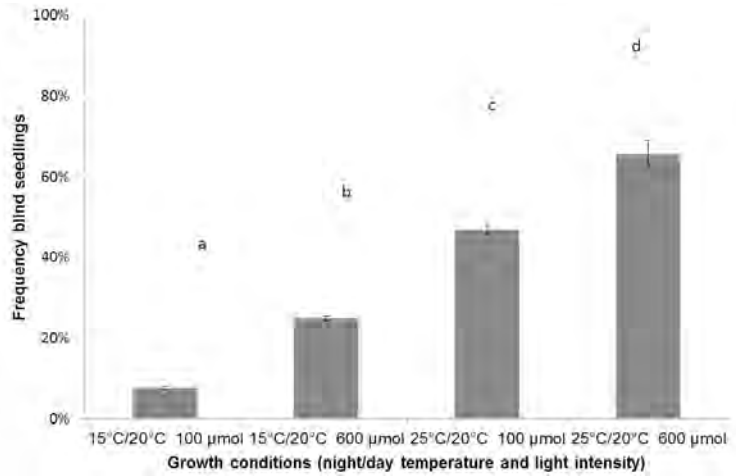

Figure 5. Frequency of blind tomato seedlings after three weeks of development at either 100 or 600µmol light, at temperatures set at 20/15 °C or 25 °C and a day-length of 16h with constant or alternating day/night temperatures. Error bars represent the standard error of two replicates of 328 seeds. Letters (a,b,c,d) represent stistical different subsets (Student's t-test, P=0.05). Averages, SE and P-values were calculated after probit transformation.

High temperature affects meristem maintenance in tomato before radicle protrusion

The previous experiments showed that high temperature affects SAM development of sensitive tomato seedlings. To obtain information about the most sensitive period and the effect of different temperatures on blindness induction during the germination phase, seeds were sown and incubated at different temperatures in darkness and then transferred to standard growth conditions with light. The tomato seeds imbibed in the dark at 33°C until radicle protrusion developed over 50% of blind plants, while seedlings imbibed at 19°C until radicle protrusion developed only 9% blind plants (Figure 6). This confirmed that elevated temperature alone can cause SAM arrest in tomato.

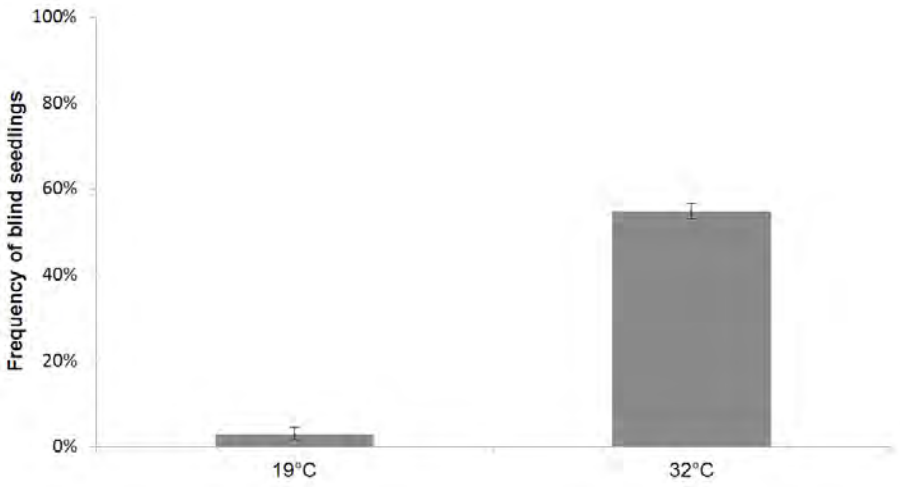

Figure 6. Effect of germination temperature on the percentage of blind 'Kinsberg' seedlings. Seeds were germinated at 19 °C or 32 °C in the dark and afterwards transferred to 16h light and 21 °C. Error bars represent the standard error of four replicates of 84 - 120 seeds. Student's t-test, P=0.002. Averages, SE and P-value were calculated after probit transformation.

To study the period during which the seeds or seedlings are sensitive to the induction of blindness, seeds were imbibed in the dark at 19°C and subjected for 24 hours to 33 °C during a period of four days (Figure 7). This means that seeds were subjected to high temperatures either at day one, day two, day three or day four. The rest of the time they were kept at 19 °C, till radicle protrusion, after which the seeds were transferred to a growth chamber with standard growth conditions (21°C, 16 h light (100 µM)). Since not all seeds germinated at the same time, some seeds were transferred at day three, most on day four and some on day five. The highest number of arrested seedlings (40%) was reached when the seeds were subjected to the higher temperature at day two after sowing, which means after having imbibed for one day at 19°C (Figure 7). However, already a 24 hour treatment of 33°C on the first day after sowing yielded 30% of blind plants. Seeds that had remained continuous at 19 °C during the germination phase gave only a very low frequency of blind plants, whereas seeds that were either the first or the second day at 33 °C, gave rise to a high frequency of blind plants. However, the sensitivity decreased if seeds were subjected to the high temperature on the third day after sowing, which is just before radicle protrusion for the majority of the seeds (T_{50} was around 75 hours). Our results suggest that for blindness induction at 33 °C the first 72 hours after

start of imbibition are the most important, because the sensitivity for temperature declines after that time (Figure 7). The non-germinated seeds transferred after three days of development from 19 °C to 33 °C showed no significant difference in the number of blind plants compared to the seeds continuously incubated at 19 °C (Figure 7).

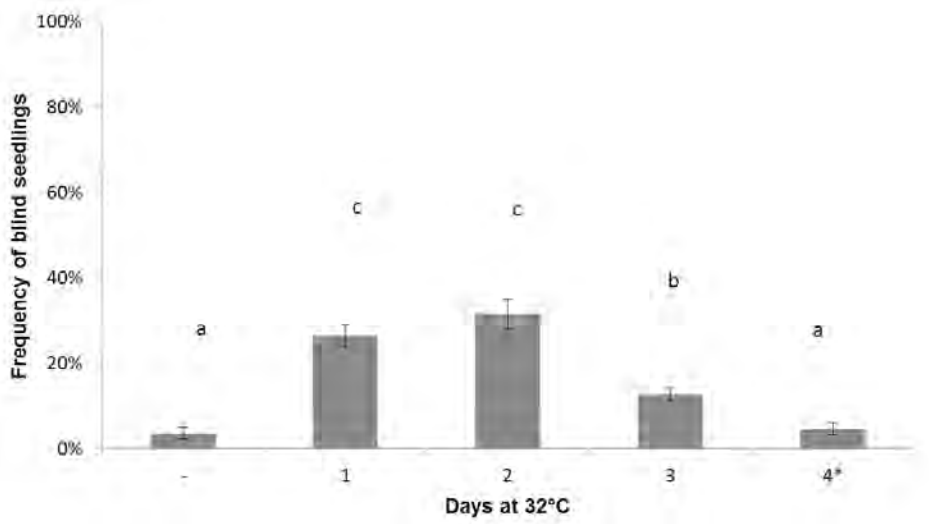

Figure 7. Frequency of blind 'Kinsberg' seedlings after subjecting the seeds for 24 hours at 32 °C at starting either at day one, two, three, or four after sowing. The most left bar (-) represents control seedlings that were not subjected to 32°C. Seeds were transferred to 21 °C and light after germination, starting after three days. Error bars represent the standard error of three replicates of 60 seeds. Except for 4* were only 24-30 non germinated seeds were available. Letters (a,b,c) represent stistical different subsets (Student's t-test, P=0.05). Averages, SE and P-values were calculated after probit transformation.

In addition to the increase of blind seedlings through high temperature incubation, also the number of plants categorized as abnormal is three times higher, although the ratio of blind to abnormal plants is the same over the treatments. One of the reasons for this increase in abnormal plants could be that the arrest of the meristem is a gradual process and may affect leaf formation before losing fully meristem activity.

It was observed that also in *Brassica oleracea* temperature stress during the germination phase leads to SAM arrest (chapter 3). In contrast to tomato, where high temperatures causing SAM arrest, in *Brassica oleracea* this arrest is induced by low temperatures. It is not known if any temperature stress triggers the same

mechanisms in both species or if low and high temperatures are perceived as different signals in brassica and tomato, respectively. Low temperatures (0-4°C), the trigger for blindness induction in brassica, does not induce blindness in tomato, which suggests that different pathways are triggered in tomato and brassica but with the same result on the induction of blindness. Alternatively, cold imbibition is not perceived as stress in tomato. Germination at 33 °C is a suboptimal condition for tomato seeds and can be seen as germination under stress (Coolbear and McGill 1990). In Arabidopsis, germination is delayed at 32 °C. This delay is regulated by FUSCA3 (FUS3) a B3-domain transcription factor (Chiu, Nahal *et al.* 2012). The *FUS3* expression is up-regulated in seeds that are imbibed for 24h upon exposure to 32 °C. This up-regulation is due to the reactivation of the promoter of *FUS3*. These results from Chiu et al (Chiu, Nahal *et al.*) indicate that temperature has an direct effect on the seed and can inhibit developmental processes such as seed germination. We found that the sensitive period for the induction of SAM arrest spans the second and third day after starting imbibition. Imbibing seeds after or before that period, result in significantly less plants with arrested meristem, demonstrating that the seeds can perceive the signal for the arrest of the meristem only in a small developmental window. Also the *FUS3* expression pattern in Arabidopsis seeds follows a tightly regulated time frame window, in which the *de novo* transcription is re-activated by high temperatures preventing seedling growth.

In general during the first day of imbibition, seeds are taking up water, the metabolic activity is initiated and the cells leave their quiescence stage (transition from G_0 to G_1 phase) (Bewley, 2013). In tomato during the second day of seed imbibition, DNA replication starts in cells located at the radicle tip meristem and the SAM, where the cell cycle progresses from G_1 , through S towards G_2 phase (Bino, Vries *et al.* 1992)(de Castro, van Lammeren *et al.* 2000). Mitosis only takes place after radicle protrusion. The period of highest sensitivity to the induction of SAM arrest coincides with the onset of cell cycle activity in these meristems, suggesting that in sensitive seeds the normal progression of cell cycle activity is disrupted by the high temperature stress.

Radicle protrusion itself is not inhibited by the high temperature stress, since the T_{50} (time till radicle protrusion for 50% of the seeds) is 96.1 hours for the seeds

germinated at 20 °C and even slightly faster (93,3 hours) for seeds germinated at 33 °C (Figure S3). Affirming that SAM arrest through high temperature is a process limited to the SAM.

Effect of seed moisture level during priming

Because we found that the sensitivity to SAM arrest in tomato is especially in the days before radicle protrusion, and because of the varying results obtained with priming (Figure 2) we were interested in the effect of seed moisture level during priming. We hydro-primed seeds for seven days at three different moisture contents and at 15°/20° (night/day temperature). Seeds primed at 35% moisture content (on wet weight basis) developed more blind plants than at either lower or higher moisture levels or the untreated samples (Figure 8). For both stresses, the seeds are especially sensitive in the first phases of germination before radicle protrusion. From previous studies on tomato seed priming, it is known that during the sensitive period of 24h-48h DNA replication is initiated in the meristems of tomato (De Castro, Van Lammeren *et al.* 2000). The absence of an effect upon hydro-priming at 30% MC, can be the absence of cell cycle initiation, or a rather slow progression of it during priming at relative low moisture levels (Van Pijlen, Groot *et al.* 1996). Confirming that SAM of tomato seeds are in a very sensitive state early during imbibition.

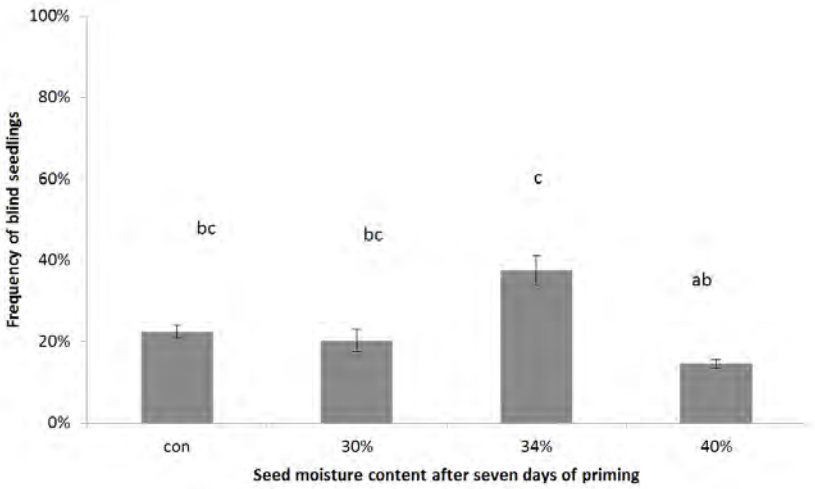

Figure 8. Priming effect on induction of blind plants (tomato cv 'Kinsberg'). Seeds received either no priming treatment or a hydro-priming treatment for 7 days at a moisture content as indicated. Error bars represent the standard error of two independent experiments with each 240 seeds per treatment.

Sensitivity for shoot apical meristem arrest is transferred to the next generation and is influenced by seed production conditions, but not by the parent phenotype

We investigated whether the sensitivity to blindness has a genetic or epigenetic base and can be transferred to the next generation. Seeds from the 'Kinsberg' F_1 hybrid seed batch were germinated at 33°C, subsequently, their seedling phenotype was scored and seedlings were transferred to the greenhouse. Some of the plants initially scored as blind produced a secondary shoot from which seeds could be obtained. F_2 seeds were produced at two different seed companies, on plants from either 'initially blind' or 'normal' seedlings. These next generation seeds were tested for their sensitivity to blindness induction. The offspring's of the sensitive F_1 hybrid plants also developed high frequency of seedlings with an arrested SAM after germination at 33°C (Figure 9). On average the plants exhibited 54% of blind plus abnormal seedlings. The sensitivity present within the F_1 hybrid seeds was retained to the same level in the F_2 population indicating that the sensitivity was inherited. No significant difference was found between seeds produced from initially blind seedlings and those from normal seedlings. This shows that the genetic background (i.e. sensitivity) and/or the production conditions are more important than the phenotype of the mother plant. If blindness is related to DNA or histone modifications, i.e. epigenetic factors are involved, then these are reset to their 'normal' state in the side shoots that escaped from the blind phenotype and behave like a non-blind parental plant. It is therefore unlike that blindness in tomato is caused by an inherited epigenetic modification. Not all tomato genotypes develop blind seedlings (Buitelaar 1995), indicating that there is at least a genetic component linked to this trait.

Blindness is definitely influenced by the environment that the plant is exposed to, because the F_2 seed lots that were produced at two different seed companies, displayed a significant difference in the frequency of blind seedlings (Figure 9). The exact reason for this is not clear, it can be due to differences in the growth conditions of the mother plant, the way of harvesting, cleaning and drying or other treatments of the seeds. Small environmental fluctuations or the age of the mother plant do not have an effect on the sensitivity as seed batches sequentially harvested from individual mother plants, showed no significant difference in sensitivity. The

plasticity of the phenomenon hints at a factor that is directly influenced by the environment. Further studies are needed to analyse the seed production effect and the mechanism of SAM arrest in tomato.

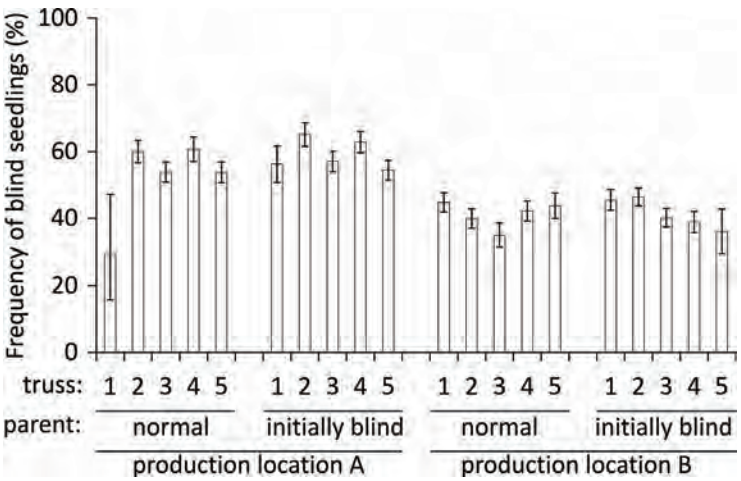

Figure 9. Frequency of blind seedlings observed in next generation (F2) Kinsberg population. Seeds were from truss 1, 2, 3, 4 or 5 of normal (0) or initially blind (1) plants produced at two locations (A or B). Error bar represents Bars represent the standard error of 15 samples of 72 seeds for location A seeds or on average 30 samples of 24 seeds for location B. Averages and SE were calculated after probit transformation.

Conclusion and remarks

Spontaneous shoot apical meristem arrest is a hardly understood phenomenon in tomato. This is probably because of its irregular and highly variable manifestation from season to season, the variation in sensitivity between seed lots and the differences in sensitivity between varieties. The large variation in the occurrence of arrested meristems, both between different seed-productions and between individual plants in a genetically uniform (F_1) population, indicates an influence of the environment on SAM arrest in tomato. In this study we were able to develop a protocol that could induce a high frequency of blind plants with sensitive seed lots. We demonstrated that seed imbibition conditions play an important role in SAM development of tomato seedlings. Both high temperatures and priming

conditions could induce an arrest in further SAM development in sensitive tomato seeds. Likely a genetic factor is involved as well, because of the large variation in sensitivity among tomato accessions. Although the exact mechanism underlying this phenomenon remains unclear, it is tempting to speculate that it is caused by an impaired (re-)activation of the SAM after the seeds are released from the quiescent state. However, the phenotype is variable and even some seedlings can produce first leaves, although these are often abnormal. The SAM arrest can be triggered by heat stress and is perceived in imbibing seeds. More research is needed to elucidate the molecular mechanism and to identify pathways regulating the SAM function in imbibing seeds. Comparing the transcriptomes of induced and non-induced seeds might identify causal factors leading to SAM arrest. The developed protocol to obtain a high percentage of arrested plants in sensitive seed lots, will be useful for genetic analysis and understanding of the physiology behind the induction of blind plants. To test if a genetic factor is involved and to identify potential genetic regions associated with the phenotype a mapping population segregating for this trait should be created and together with the induction protocol, QTL analysis for variation in sensitivity should be performed.

Material and Methods

Seed samples

A seed batch from the tomato Kinsberg F_1 was provided by Rijk Zwaan Zaadteelt en Zaadhandel B.V. (De Lier, The Netherlands). Additionally, next generation seeds from this F_1 hybrid were produced at different locations and analysed for sensitivity. Seeds from Variety 3 were provided by Nunhems B.V. (Nunhem, The Netherlands) and from variety Oscar by ENZA Zaden Seed Operations B.V. (Enkhuizen, The Netherlands).

Analysis of the effect of light intensities on the development of blind seedlings

Seedling development experiments were carried out in phytotrons at 25 or 20 °C, and set with either 50, 100, 300 or 600 µmol light for 16 hours a day (Osram and Phillips 80W T5 High Output). Tomato seeds were sown on rock-wool plugs (40

x 40 mm, 6/15 holes) placed in trays (30 x 50 cm) with cotton sheets under the plugs. The plants were watered three times a week with a standard tomato nutrient solution. Experiments were carried out in triplicate in a randomized block design. Three weeks after sowing the phenotype of seedlings was analysed.

Temperature germination assay

Tomato seeds were sown on rock-wool blocks in nutrient solution and placed in incubators set at either 33°C or 19°C in the dark. Rock-wool blocks with germinated seeds (radicle protruded) were transferred to growth chambers with 16 h of light/8 h of dark (100 µmol) at 21°C on a daily basis starting from day 3. After three weeks seedlings were classified into the categories as described above. Trays were watered three times a week with standard tomato nutrient solution.

Seed priming

500 mg of seeds were hydro-primed in 50 ml tubes with water until the desired moisture content (wet seed based). Tubes were sealed with oxygen permeable polyethylene foil. The seeds were incubated for seven days on a roller bench at either 19°C or 25°C in the dark. When priming was finished, the seed were dried for at least three days on filter paper in an incubator at 20° with a relative humidity of 32%. Afterwards the primed seeds were sown in rock-wool blocks as described above and grown in the same growth chamber with the same nutrient solution as described above. Priming by the seed companies was performed according to their non-disclosed commercial protocols.

Seed production of F_2 seeds and testing of the seeds

At two locations, F_2 generation seeds were produced from F_1 plants (c.v. Kinsberg), from 15 - 30 normal plants and 15 - 30 plants that were initially blind but produced a secondary main shoot from an axillary meristem. Seeds were separately harvested per individual mother plant and fruit truss. Seeds were stored at 32% relative humidity and 20 °C until the testing. Seeds were sown in rock-wool blocks and first incubated at 30°C in the dark and 100% relative humidity for three days and subsequently in the greenhouse at 25°C for 18 days. Trays were watered every two days with tomato nutrition solution. Seedlings were phenotyped according to the categories described above after two weeks of development.

Acknowledgements

Corine de Groot, (Bejo Zaden B.V.) and Ronald Driessen (Rijk Zwaan Zaadteelt en Zaadhandel B.V.) are acknowledged for their contribution in optimisation of the assay to determine the sensitivity of seed lots and induce the development of blind plants.

Supplement

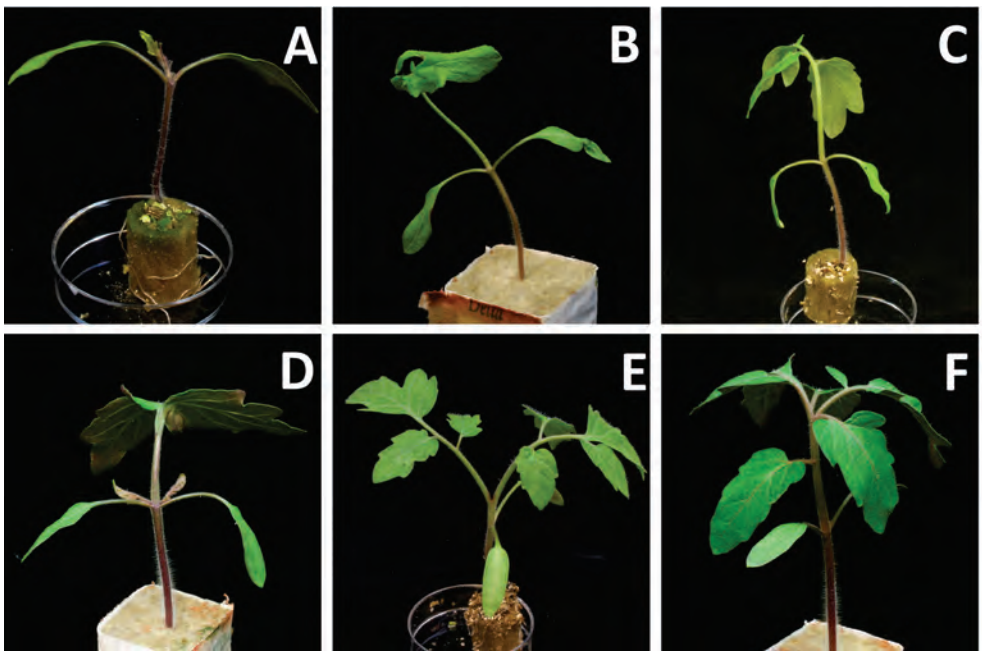

Figure S1. Variation of the blind phenotype in tomato. A: Three weeks old tomato seedling with side shoot growing from the axil of the cotyledon, B: Seedling with a leaf like structure, C: Seedling with one normal leaf, D: Seedling with one leaf and two side shoots growing from the axils of the cotyledons, E: Seedling with two leaves but no internodes, F: Seedling with one normal leaf and one aberrant leaf without internodes.

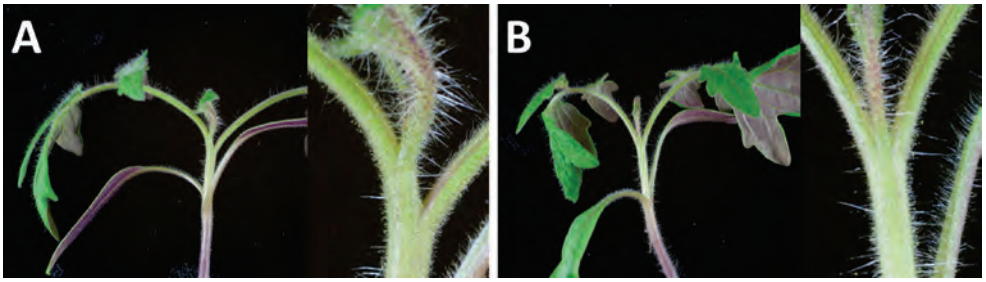

Figure S2. Three weeks old tomato seedling of the variety Kinsberg. '. A: Normal developed shoot and in B: Abnormal tomato seedling with aberrant positioning of the shoot.

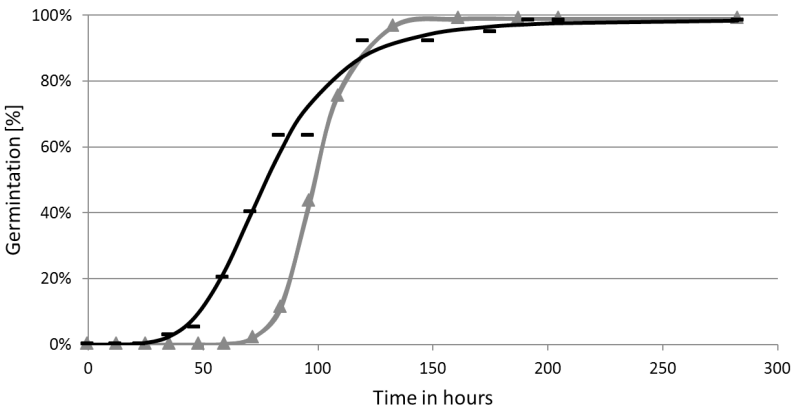

Figure S3. Germination curve of tomato seeds of the cultivar 'Kinsberg', upon germination at 20 °C (Δ) or 33 °C (-). T50 at 20 °C 96 hours. T50 at 33 °C 93 hours.

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Conclusion and General Discussion

Jennifer de Jonge

Chapter 5

Introduction

In this thesis I have studied the phenomenon of spontaneous SAM arrest in brassica and tomato seedlings. I investigated the environmental and genetic causes for this SAM arrest and described the morphological differences between normal and so called blind seedlings (Chapter 2 and 4).

For *B. oleracea* cold imbibition was identified as a blindness inducing factor. With this information an efficient assay was developed to screen genotypes for blindness sensitivity. If the seed-moisture content of initially sensitive brassica seeds was increased by imbibition at room temperature, these seedlings will subsequently be resistant to the inductive conditions in the assay (Chapter 3). However, this pre-treatment only works in a limited range of seed moisture contents (Chapter 3). To identify the potential genetic component involved in SAM arrest, the developed induction assay was used together with a quantitative mapping approach, which resulted in the identification of one region associated with blindness sensitivity, located on chromosome three (Chapter 2). Furthermore, I discovered that the cell cycle of SAM cells in sensitive brassica is abnormal and DNA replication is abolished, possibly due to down-regulation of an MCM2-3-5 homolog after cold treatment in a sensitive genotype (Chapter 2).

While brassica seeds respond to low temperature, tomato seeds develop blind seedlings when the imbibition temperature is too high (Chapter 4). Both species have in common that they respond to temperature stress during the imbibition period (Chapter 2 and 4).

In this chapter I will discuss the phenomenon “blindness” in general and what may lead to this aberrant development that plants suffer from SAM arrest. Furthermore, I will speculate about the mechanism of increasing the seed moisture content of brassica seeds and its narrow window of effectiveness. Additionally, I will point out the practical implications of the results obtained in my thesis and how these can help the seed industry. Future experiments that could help to unravel the scarcely studied phenomenon of SAM arrest are discussed.

Phenotypic plasticity, domestication and environmental responses

Plants are sessile organisms and therefore exposed to the environment and its fluctuations in biotic and abiotic factors. Consequently, plants express different phenotypes in diverse environments. In order to deal with this situation, plants evolved complex signalling and protection mechanisms. This evolution of phenotypic responses to the environment is also known as phenotypic plasticity (Gause 1947). The ability of brassica and tomato plants to imbibe at unfavourable temperatures and still being able to germinate afterwards could be part of their phenotypic plasticity. There are theories stating that phenotypic plasticity may entail fitness costs or trade-offs, which is a fundamental feature of ecological strategies. (DeWitt, Sih *et al.* 1998). Given finite resources, organisms must choose to allocate these resources between growth, maintenance and reproduction on the one side and response to environmental stress conditions on the other side. Plants encounter a large number of trade-offs during their life cycle, such as seed size/number trade-off (Leishman 2001), colonization-competition trade-off (Cadotte 2007), and the growth-defence trade-off (Thaler, Fidantsef *et al.* 1999). In the case of brassica and tomato plants, this cost of plasticity could be the trade-off between germination at unfavourable conditions (e.g. low or high temperature) and meristem arrest, which impairs further development.

The costs of plasticity are usually expressed as the trade-off between tolerance breadth and fitness under optimum conditions (Chevin, Lande *et al.* 2010). Therefore, these blind-sensitive genotypes may have a better fitness or another favourable trait that make them more useful as commercial plant variety. In other words, the trade-off concept is linked to a specific trait and not necessarily related to the overall fitness of the plant.

In nature, where the environment changes constantly, organisms are challenged to maximise their total fitness under these heterogeneous conditions, which is done by natural selection leading to environmental canalization. This canalization reduces the influence that the environment has on the expression of a trait and increases phenotypic plasticity (Auld, Agrawal *et al.* 2010). Conversely, in breeding programs, seed companies select elite genotypes containing specific favourable

traits. In particular they select their varieties for optimal growth and performance under conditions practiced by farmers. In the case of cauliflower it is general practise in the Netherlands to sow brassica seeds in the winter in unheated greenhouses, in order to have a harvestable product early in the year and to avoid the so called “white weeks” in which the prices for cauliflower drop tremendously. These conditions are not optimal for plant development and in this thesis it has been shown that imbibition temperatures of four degree can induce SAM arrest in susceptible varieties. For example a very sensitive kohlrabi seed lot showed already high frequencies of blind seedlings after the seeds were imbibed for just a couple of days at four degree. The varieties are in this case selected for their ability to germinate after a long period of cold. The process in which human or animal use of plant species lead to the selection of physiological and morphological phenotypes that allow us to distinguish those plants from their wild ancestors is called domestication (Hancock 2005). The main developmental traits selected for, in both tomato and brassica varieties, are apical dominance, homogeneous growth, flowering and fast germination. In general, domestication often involves an increase in apical dominance; a typical example is the domestication of the maize ancestor teosinte, where it involves the suppression of axillary branching and the allocation of resources to the main stem (Doebley, Stec *et al.* 1997). Apical dominance is established by the plant hormone auxin that is synthesized in the shoot apex and moves down the stem, inhibiting the outgrowth of axillary buds (Leyser 2005). Therefore, higher auxin levels could lead to easier farming of the crop, for example less pruning of axillary shoots, but might have a disadvantage for the response to stresses (e.g. low temperature) at the same time, because auxin homeostasis and signalling are known to be influenced by cold (Lee, Henderson *et al.* 2005).

SAM arrest is described in Brassica since 50 years. Selection for advantageous traits, adapting the crop to conventional farming systems might be a possible reason for the introduction of SAM arrest, because all studies published so far describe the occurrence of this phenomenon in varieties bred by breeding companies. Additionally, the resistant broccoli parent of the AGDH population (Chapter 2) originates from a conventional broccoli variety ‘Green Duke’. Also no reports are known that describes the blind phenomenon in wild relatives, which might be

related to the lack of studies in wild relatives or alternatively, it might point to a link with domestication. When we studied the model plant *Arabidopsis thaliana,* which belongs to the Brassicacea family and is therefore closely related to *broccoli,* SAM arrest could not be induced. The common ancestor of *A. thaliana* and *B. oleracea* diverged only five million years ago (Parkin, Gulden *et al.* 2005) and brassica genes share a high level of sequence similarity with their Arabidopsis orthologous (Cavell, Lydiate *et al.* 1998). Taking into account that Arabidopsis is a weed and has not been domesticated, this also underlines the hypothesis that domestication increases the sensitivity for blindness in crops.

During development of the blindness preventive treatment, an influence of the brassica seed moisture content on the frequency of blind seedlings was discovered. Therefore, we believe that something needs to be present in dry seeds leading to arrested meristems if imbibed in the cold (Figure 2). If the seed moisture content is elevated to ten per cent at room temperature the cold has almost no effect (Chapter 3). However, this sensitivity reducing effect of pre-soaking could only be preserved in re-dried seeds if the seed moisture content was elevated to approximately 20% (Chapter 3), but elevating it up to fully imbibed (50% MC) increases the sensitivity again. However, if the seeds are pre-soaked in an excess of water (>50% MC) re-drying cannot re-induce blindness sensitivity. It is possible that the sensitivity factor, if existing, is removed by washing in an excess of water. To identify what is happening in the seed and to unravel processes that decrease and increase the sensitivity for blindness, protein, metabolite and transcriptome analyses could be conducted. These “omics” experiments should be conducted at the relevant seed moisture contents of the seeds, with and without cold treatment of a resistant and sensitive genotype. By following the state of the molecular processes involved over time we should retrieve more information about the processes happening during blindness development in the seed. However the comparison of a resistant with a sensitive genotype will be difficult as the sensitive genotype never results into 100 per cent blind seedlings.

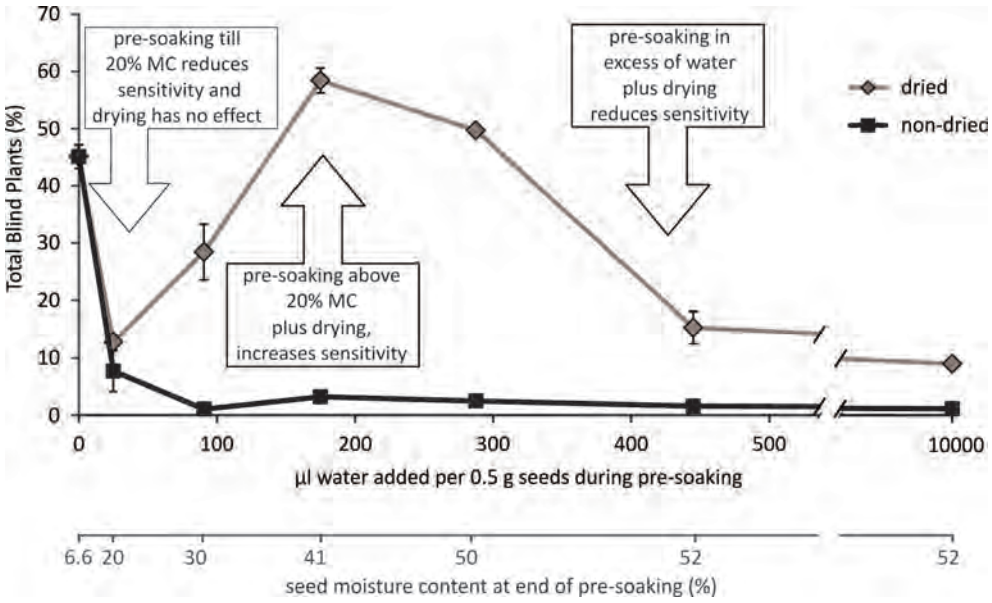

Figure 1. Effect of a pre-soaking treatment, at 20 °C and with addition of different amounts of water, on the subsequent sensitivity of the seeds to cold incubation and development of blind seedlings. The pre-soaking was followed by either direct incubation in the cold assay, or a re-drying step between the pre-soaking and the cold assay. Interrupted lines mean pre-soaking seeds in excess of water without a significant increase in seed moisture level.

Also the physiology of sensitive tomato seeds exposed to different imbibition periods should be analysed in more detail to understand processes resulting in high frequencies of blind seedlings. So far, not many studies have been conducted, where the first 24 hours of imbibition were shown to have an effect on the SAM of a young seedling. Most of the published literature concerns the analyses of abiotic stresses and their effect on the root meristem. This could be due to the fact that the root is easier accessible in seeds or that until now, effects of seed-imbibition conditions on the SAM have not been found. In this thesis we found that seed-production or treatment conditions influence the sensitivity of the tomato seeds to meristem arrest if imbibition temperatures are high (Chapter 4). Also seed companies observed differences in sensitivity of tomato F_1 hybrid seeds produced in different countries (Chapter 4). To identify which factor during seed production or treatments causes the sensitivity, new experiments need to be designed. This is complex because seed-production is a process in which various factors can be changed and analysed. For example the environment in which the mother-plant

develops, e.g. temperature during flowering, fertilization or fruit development. Next to the effect of temperature, the humidity, light intensities, and nutrition of the mother-plant are often variable and could have an effect on SAM vigour in the seed. Seed-production however, also involves the harvest of the fruit, seed cleaning from the flesh and drying of the seeds. All these factors should be tested separately in carefully designed experiments, preferably with a genetically fixed line that is sensitive to SAM arrest. As mentioned earlier in the discussion, we obtained indications that the ripeness of the fruits influences the sensitivity; therefore one of the first future experiments could be to harvest the fruits at different ripeness and testing for their sensitivity.

Practical application of the results obtained in this thesis

When starting this study, the information available about the cause for shoot apical meristem arrest in tomato and brassica was very limited. Therefore, we collected information about potential conditions that were suspected to be the cause for this arrest, from experts in the Dutch seed and plantlet industry (Chapter 1). During this study we were able to eliminate some of these suspected conditions. SAM arrest could not be induced in *Brassica oleracea* plants, when grown under molybdenum deficiency. No influence was found of mineral nutrition in general and also not after the application of blindness inductive conditions. Furthermore, during the cold induction light did not have any effect on the percentage of seedlings with arrested meristems. Cold during the seed germination phase, prior to radicle protrusion, was shown to be the main trigger in inducing blindness in sensitive brassica seed lots.

The cold imbibition assay can now be used as screening tool at the seed companies, to test for potential blindness sensitivity in their new varieties or seed lots and to ensure high seed quality. Additionally, the blindness preventing treatment (Chapter 3) can now be used for varieties or seed lots that have a potential commercial value, but contain the risk of developing blindness when the conditions are unfavourable.

Unlike brassica, tomato seedlings have normal meristems if they are imbibed at low temperatures but their seeds respond to high temperature with blindness. During the studies presented in this thesis light could not be excluded as factor

causing blindness but most likely it is temperature alone that affects the meristem. Both hormones and light have shown to affect meristem activity and maintenance in Arabidopsis (Yoshida, Mandel *et al.* 2011). However, no effect of hormones on SAM arrest was detected in tomato seedlings, at least not in the concentration and the hormones tested. Only one study reports about the cause of SAM arrest in tomato but with inconclusive results (Wetzstein and Vavrina 2002). In this thesis germination at high temperatures was identified as condition leading to SAM arrest in seedlings. These imbibition conditions can now be used by the seed companies as a fast and easy test to screen their seed lots, new varieties and crosses for their sensitivity to blindness. Additionally, the seed companies are now aware of the fact that seed production conditions may have an influence on the sensitivity to SAM arrest in the next generation, which was not even suspected to be a potential cause. One of the causal factors could be a too early harvest of the tomato fruits, because a pilot experiment showed that seeds harvested from unripe tomato had more arrested seedlings than seeds that originated from ripe fruits.

Not only tomato and brassica suffer from SAM arrest, but also other crop species such as bell pepper (Chapter 1). Tomato and bell pepper are both Solanaceous crops, whose wild forms originate from the same geographic region and share similar domestication history (Paran and Van Der Knaap 2007). Therefore, it could be possible that they respond to the same environmental conditions. Given the fact that both crops have similar domestication histories and that both suffer from SAM arrest this could be another indication for the involvement of domestication in the development of blindness. To test whether the tomato conditions can also trigger SAM arrest in bell-pepper, the same assay as for tomato should be applied to sensitive bell-pepper seeds. Together with other experiments this may provide other clues that the (genetic) mechanism behind SAM arrest is conserved between different species.

Future experiments** to **unravel the molecular genetic mechanism that leads to SAM arrest in tomato and brassica

The fact that sensitive and resistant genotypes exist, points to a genetic factor that is involved in meristem arrest in both species (Chapter 2 and 4). The short and effective assay that was developed to induce blindness in sensitive brassica genotypes was very useful to obtain molecular and genetic information of genes and loci underlying this phenomenon (chapter 2). The QTL analysis revealed genetic variation of this trait on chromosome 3 and combined with transcriptome analysis, a list of potential candidate genes was selected. Among these candidate genes, a member of the MCM2-3-5 family was found and down-regulated after the cold treatment in sensitive seedlings. This gene is involved in DNA replication initiation and therefore essential for fast dividing cells. Possibly, other genes involved in the core cell cycle, such as CYCLINS are down-regulated as a secondary response in 7-days old seedlings. Further studies are needed to assign a causal relationship between these candidate genes and the blindness phenotype. To confirm this relationship one could test the expression levels of the candidate genes at different moments of blindness induction. Also sequence differences between the sensitive and the resistant genotype could be analysed to identify potential SNPs in promoter or coding region, which could change the expression or function of the gene and hence arrest the meristem. Functional assays could be done in a heterologous system, such as Arabidopsis. The candidate genes can be introduced in Arabidopsis or, alternatively, Arabidopsis orthologs could be mutated and the effect on SAM activity investigated.

A similar approach as the one in Brassica could be followed to identify the genetic base for SAM arrest in tomato and to discover the underlying gene(s). For this, a mapping population segregating for this trait needs to be available. As a first step, parents of publicly available tomato populations should be tested for genetic variation in sensitivity with the high temperature germination protocol. Subsequently, QTL regions could be identified and together with the genome sequence, this approach should lead to a list of candidate genes. It will be very interesting to compare these candidates from brassica and tomato and to see if there is a common mechanism for meristem arrest in both species. As an alternative to conventional QTL analysis, also Genome Wide Association Studies (GWAS) is coming into reach, since many tomato genotypes have been sequenced recently (see 150 tomato genome project; http://www.tomatogenome.net/)

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Summary

The life cycle of a plant starts with the fertilised egg cell, soon followed by the first divisions and polar differentiation and the formation of apical meristems in the embryo. This embryo resides in a seed that germinates into a seedling which unfolds its cotyledons and produces most of the above ground tissues from the shoot apical meristem (Yanai, Shani *et al.*). A pool of cells known as stem cells located in the centre of the shoot apical are responsible to maintain meristematic activity throughout a plants life in order to produce organs. The maintenance of these stem cells is tightly controlled by a complex genetic and hormonal network. Any disruption that leads to the loss of stem cells will end the formation of new plant organs and therefore the plants life-cycle. The balance between leaf initiation and meristem maintenance is controlled by internal and external factors, although our knowledge about the nature of these factors is very limited.

This thesis reports the results of a study on SAM loss in tomato and brassica and the genetic and environmental factors causing this arrest. The aim was to study which environmental conditions could lead to so-called blind tomato and brassica plants and to develop a method that could induce this phenomenon. Furthermore, we aimed to understand if a genetic factor is involved in the sensitivity to blindness.

Literature on SAM arrest is very limited and therefore this research was started by interviewing specialists in the field, to learn more from their experience with SAM arrest. The experts stated that next to temperature, also light intensities and the imbalance of light and temperature could cause SAM arrest in tomato (**Chapter 1**). Therefore, the thesis focussed on the effect of temperature and light intensities on the maintenance of the SAM in tomato and brassica. **Chapter 2** describes blindness in brassica and which environmental and genetic factors play a role. It was observed that the stage of development at which the arrest of the SAM occurs is variable and blind plants can vary in their appearance from no leaves to plants with a few leaves, although these leaves are often aberrantly shaped. Some arrested Brassica seedlings have the ability to generate new meristems from the axils of their cotyledons. The tunica-corpus structure present in the apex of normal seedlings is absent in blind ones.

We identified that subjecting brassica seeds of sensitive genotypes to low temperatures (0-3 °C) at early stages of germination could induce blindness. Using this knowledge we developed an assay that was applied to several different brassica lines and varieties. Because these plants showed different frequencies of blind seedlings in response to the treatment, it was concluded that a genetic factor is involved in blindness sensitivity. Subsequently, we screened a mapping population segregating for the trait and a significant region on chromosome three, associated with the occurrence of blind seedlings was identified. Since many genes were underlying this QTL region, a transcriptome analysis was conducted, with and without a cold treatment on sensitive and resistant lines. This analysis yielded about 40 genes that were located in the QTL region and also differentially expressed between induced and non-induced seeds. Among these candidate genes is a member of the *Mini Chromosome Maintenance (MCM)2-3-5* gene family. These genes are involved in initiation and prolongation of DNA replication in eukaryotes and are consistent with its role in DNA replication, preferentially expressed in young tissues that contain a high number of replicating cells, like embryos, young organs and meristems. Other cell cycle related genes were also affected and miss-regulated in the induced seedlings, but these genes were not found in the QTL region and are therefore most-likely indirectly affected. Evidence that the cell cycle was impaired in the arrested seedlings came also from experiments with EdU staining that marks nuclei in the DNA replication phase. Whereas the dye was incorporated in the DNA of SAM cells of seedlings of the resistant line the staining was much lower in SAM nuclei of the sensitive seedlings.

Knowing that imbibition and subsequent incubation at low temperature is a key stress factor for the development of blind seedlings it was important to further investigate the effects of water temperature during initial seed water uptake (**Chapter 3**) . In this chapter we describe that a pre-soaking procedure on blindness-susceptible kohlrabi and cabbage seeds in water of 20 °C prior to the cold imbibition for two hours, can effectively prevent cold-induced blindness. This pre-soaking effect that reduces the sensitivity of the seeds to blindness induction is affected by the period of pre-soaking, the moisture content during the soaking and the drying after the soaking. We hypothesize that DNA-damage in the seeds that occurs during

seed drying and storage is repaired during the pre-soaking imbibition phase at room temperature. The cold treatment alone appears to be unable to repair the damage and leads to arrested seedlings.

Also in tomato blindness occurs and is described in **Chapter 4.** In tomato, SAM arrest most often occurs after the initiation of two leaves. In comparison to a normal plant, tomato plants with an arrested apex can often have only one or two leaves that are fused together at the stem. Once arrested, plants may remain without a shoot and further leaf development. However, some plants are able to form new shoots from axillary meristems present in the axils of the cotyledons or the first leaves. These side-shoots develop further normally and are able to flower and produce seeds. Also in tomato blindness frequencies depend on the temperature during incubation, but here it is a relative high temperature that induces the formation of blind plants in sensitive seed lots. The observed blindness inducing effect of high light intensities is most likely mediated by an increase of the temperature at the seed level since high temperatures can also induce blindness when seeds are imbibed in the dark. The sensitivity window for high temperature induced blindness in tomato is restricted to the first two days after wetting the seeds, which is prior to root protrusion.

Priming is a common seed-treatment for tomato seeds, used to promote or synchronises germination of a seed-batch and performed by controlled hydration of seeds followed by drying. We observed that primed seed-batches can show a higher percentage of blind plants than untreated seeds. Furthermore, seed lots of the same F_1 hybrid, produced in three different countries, showed different frequencies of blind seedlings. Also F_2 seeds produced at two seed companies from the same F_1 hybrid, differed in their sensitivity to develop blind plants. These results indicate that priming and the history of the seed lot can influence the sensitivity for blindness in tomato seeds.

In **Chapter 5**, the main conclusions and the implications of our results on the common practise of seed companies are stated. With the research presented in this thesis we have shown that SAM arrest in brassica and tomato is connected to differentiation of cells in the SAM and that this arrest can be induced by imbibing sensitive seeds at stress-full temperature conditions. The imbibition conditions can

now be used by the seed companies as a fast and easy test to screen their seed lots, new varieties and crosses for their sensitivity to blindness. Furthermore they have now the ability to treat sensitive brassica seeds against SAM arrest and they know that they have to be careful during tomato seed-production and with their priming recipe. Furthermore, we have identified candidate genetic factors that may influence the sensitivity to blindness with brassica crops. This is a very good starting point to discover the gene responsible for blindness sensitivity and to unravel the mechanism underlying the blindness phenomenon.

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First of all I would like to thank my Family who has always supported me and who helped me throughout my life. It is because of you that I was able to come so far. Thank you very much, Karin, Kees, Mandy and of course Arik and Silvia, who also gave me a second home in the Netherlands.

I still remember when everything began; it was the day that I walked out of the office of my internship supervisor Ad van den Nieuwenhuizen at Rijkzwaan. He had just told me that I should do a PhD instead of working in a breeding company. He told me that I have a scientific mind and that I should be working on my own projects in a company. Hence, I should finish my PhD and come back. Ad is probably the first person that I have to thank because without Ad I would have never thought about doing a PhD. Another advice from Ad was, if you want to work in a company afterwards, make sure that you still see and work with plants. This was one of the reasons why I chose this project, next to the fact that it involved working with my favorite vegetables broccoli and cauliflower. Therefore I want to thank Ad for his advice, you knew before me that I would fall in love with Science.

You put the seed in the ground.

Against all my expectations in the beginning, I did fall in love with Science and am now following the dream of being able to become a Scientist for the rest of my life. One of the reasons for this love is the scientific work that Gerco and the rest of the Plant Developmental Cluster are carrying out. It opened a whole new world of possibilities in Plant Science to me and created the desire to learn and use these techniques myself. Therefore, I would like to thank Gerco for inspiring me with Science and being an example for me. I also would like to thank Steven my supervisor for giving me the opportunity to be part of this group and for choosing me to carry out this project.

You watered the seed and made it germinate.

Special thanks go to Richard, who was not officially planned to participate in this project but who ended up playing a big role in it. Richard, I really thank you for being such an inspiration and for being this amazing person that you are. You give help and you stir people in the right way. You know how to motivate and be critical at the same time. It was really a pleasure being taught by you and I will always be thankful for that. I definitely learned a lot from you that I will implement in my own life.

I also want to thank Jan who basically was part of almost all experiments, which often meant looking at hundreds of plants and writing down strange short-cuts that only we could understand.

Next to the members of the project team I am also left with great memories from the whole Plant Developmental Cluster group. You are really special and I had a lot of fun with you all, especially on those crazy day-outs that involved fleeing from men with chainsaws to simulating helicopter-jumps.

Of course I would also like to thank my fellow PhD students, Sela for his endless optimism, Alice for being a colleague and very good, dear friend, Cezary for all the endorphins and adrenalin that we filled our blood with during climbing, it really made my life so much happier, Anneke, who is always in for dressing up, costumes and a good laugh, Juliana, Lucas, Livia, Diego, Jose and Denis for bringing the Brazilian spirit to the lab and also Hilda, Leonie, Suraj, Violeta, Suzanne and Hui.

You all made the seedling stronger and let it develop its first leaves.

Finally, I would like to thank also some people outside of the group and the University. For most of the fun on Friday evenings and outside work I would like to thank, Aina, Tila, Ana-Carolina, Jaqcui, Charles, Padraic, Renake, Cris and Julio. Aina for living with me and sharing opinions about basically everything in life, Tila for giving Hunter a best buddy, good advice and friendship and Ana-Carolina for being a dear friend.

Hopefully we will all see each other many times again, maybe in Sweden, maybe in Brazil!

To close, I would like to thank Matheus, who somehow still fell in love with me although I was in this crazy “I have to finish my thesis mode”. Thank you Matheus for entering my life and being by my side! Against all odds ;)

Saudade de voces todo!

With great love!

Jennifer

About the author

Jennifer was born on the 19th of March 1984 in Hannover, Germany. She was raised in the suburbs of Hannover where she finished her high school in 2003 and developed her passion for sports. Her Bachelor studies were completed in Horticultural Sciences 2007 at the Leibniz University of Hannover. For the Master she moved to the Netherlands and graduated in Plant Science 2009 with the specialization Breeding and Genetics. After the Master she became a PhD student in the group of Prof. Dr. Gerco Angenent at Plant Research International. In October 2013 Jennifer started a Post-doctorate at the Swedish University of Agricultural Science in the group of Prof. Dr. Lars Hennig.

List of publications

To be submitted publications

J. de Jonge, F. D. Goffman, J. Kodde , G.C. Angenent and S. P.C. Groot:"**A seed treatment to prevent blindness in brassica (*Brassica oleracea* seedlings)**"

J. de Jonge, J. Kodde , S. P.C. Groot, R. Immink and G.Angenent:"**Temperature affects meristem maintenance in *Brassica oleraceae***"

Submitted publications

J. de Jonge, Kodde J., Angenent G.C. and Groot S.P.C. "**High temperature induces shoot apical meristem arrest in tomato (*Solanum Lycopersicum*)"**.

Education Statement of the Graduate School Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Jennifer de Jonge
Date: 16 December 2013
Group: Molecualr Biology, and Biosciences, Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Genetic and environmental influences on shoot meristem development	Apr 16, 2009
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
2nd European Retreat of PhD Students in Experimental Plant Sciences, Cologne (Germany)	Apr 15-17, 2010
ExPectationS (Career Day Event, EPS), Wageningen University, Wageningen, NL	Nov 19, 2010
Phd student days 2011, Wageningen University, Wageningen, NL	May 20, 2011
ExPectationS (Career Day Event, EPS), Wageningen University, Wageningen, NL	Nov 18, 2011
4th European Retreat of PhD Students in Experimental Plant Sciences, Norwich (UK)	Aug 15-17, 2012
Phd student days 2012, University of Amsterdam, Amsterdam, NL	Nov 30, 2012
ExPectationS (Career Day Event, EPS), Wageningen University, Wageningen, NL	Febr 01, 2013
► **EPS theme symposia**	
2010 Theme 1 'Developmental Biology of Plants', Wageningen University	Jan 28, 2010
2010 Theme 4 Genome Plasticity', Wageningen University	Dec 10, 2010
2011 Theme 1 'Developmental Biology of Plants', Leiden University	Jan 20, 2011
2012 Theme 4 Genome Plasticity', Wageningen University	Dec 14, 2012
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting 'Experimental Plant Sciences, Wageningen, The Netherlands	Apr 06-07, 2009
NWO-ALW meeting 'Experimental Plant Sciences, Wageningen, The Netherlands	Apr 19-20, 2010
NWO-ALW meeting 'Experimental Plant Sciences, Wageningen, The Netherlands	Apr 04-05, 2011
NWO-ALW meeting 'Experimental Plant Sciences, Wageningen, The Netherlands	Apr 02-03, 2012
NWO-ALW meeting 'Experimental Plant Sciences, Wageningen, The Netherlands	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
Dr. Bruno Mueller	Nov 28, 2009
Daniel Schubert	May 11, 2010
TTi Networking event	Sep 22, 2010
Peter Cook Transcription factories as organizers of the genome; the role of fixed polymerases	Oct 27, 2010
Kirstin Bomblies "Genetic incompatibility and the plant immune system"	Nov 18, 2010
Plant science Seminar	Jan 11, 2011
TTi Networking event	Sep 21, 2011
Carol Wagstaff "crop improvement, food processing and their relationship to human health"	Oct 27, 2011
Angus Buckling "Bacteria-phage evolutionary ecology: lab, wild and applications"	Oct 20, 2011
Nuclear structure and gene expression	Nov 09, 2011
Steven Penfield "Parenting in plants: maternal control of seed dormancy "	Jun 12, 2012
Keys seminar on Seed production: "Dissection of seed development John Harada" and "Florian Harade From many countries – to many countries. Corn seed production" at KWS.	Sep 24, 2012

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TTi Networking event	Sep 19, 2012
► **Seminar plus**	
► **International symposia and congresses**	
International Symposium Auxins and Cytokinins in Plant Development, Praque, Czech Republic	Jul 11-14, 2009
6th International Symposium on Brassica and Crucifer genetics, Catania, Italy	Nov 12-16, 2012
► **Presentations**	
Poster European PhD plant retreat	Apr 16, 2010
Presentation TTI green genetics networking event	Sep 22, 2010
Presentation PhD school on plant development Retzbach germany	Oct 06, 2010
Poster TTI green genetics networking event 2011	Sep 21, 2011
Presentation at Escola Superior de Agricultura "Luiz de Queiroz" Department of Biological Sciences (LCB) University of São Paulo	Apr 24, 2012
Presentation at Depto Biologia Vegetal, IB CP 6109 UNICAMP 13083-970 Campinas, SP, Brasil	Apr 03, 2012
University of Copenhagen, Department of Plant and Environmental Sciences	Aug 16, 2012
4th European Plant Science Retreat For PhD students	Aug 11, 2012
6th International Symposium on Brassica and Crucifer genetics	Nov 13, 2012
Faculdade de Ciencas Universidade de Lisboa	Jan 13, 2013
► **IAB interview**	Feb 18, 2011
► **Excursions**	
Monsanto B.V.	Jan 22, 2011
Subtotal Scientific Exposure	*22.3 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Summer School Environmental Signaling	Aug 24-26, 2009
ETNA PhD Summerschool Genomics and Bioinfromatics	Sep 12-18, 2010
PhD school on plant development, Retzbach, Germany	Oct 06-08, 2010
► **Journal club**	
Journal club of the PRI cluster Biosciences	2009-2013
► **Individual research training**	
Subtotal In-Depth Studies	*6.6 credits**

4) Personal development	*date*
► **Skill training courses**	
PhD Assesment	2010
Effective Behavior in proffessional surroundings	Oct 2011
Scientific writing	Mar 2012
How to write a world-class paper	Oct 26, 2010
► **Organisation of PhD students day, course or conference**	
ExPectations Career Day Event	Nov 19, 2010
ExPectations Career Day Event	Nov 18, 2011
► **Membership of Board, Committee or PhD council**	
Member of PhD housing comitee	2010
Member of PhD council	Jan 2010 - May 2011
Head of the PhD council	May 2011 - May2013
Subtotal Personal Development	*10.4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**41.8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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