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Molecular characterization and analysis of a putative *AtDCN*1 T-DNA insertion line

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I declare that I have worked out this diploma thesis myself using only the literature stated.

Vienna, May, 2009

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Finally I want to thank my family, for all the possibilities they have created for me, for their deathless support and personal reflection. I love you all!

2. ZUSAMMENFASSUNG

Das Proteom von Pflanzen zeigt bemerkenswerte Plastizität, welche es ihnen ermöglicht auf Umweltbedingungen zu reagieren und Entwicklungstransitionen auszuführen (Hosp 2009).Unsere Gruppe isolierte und analysierte ein *Nicotiana tabacum* Gen (DEFECTIVE IN CULLIN NEDDYLATION 1 (*DCN*1)), ein Hefe (*Saccharomyces cerevisiae*) und Wurm (*Caenorhabditis elegans*) Homolog, welches Ubiquitin und RUB/NEDD8 bindet (Hosp 2009). Zusätzlich assoziiert es mit Cullins und einem NEDD8 E2-konjugierendem Enzym (Hosp 2009). Auf Cullin basierende Ubiquitinligasen werden durch Modifikation von Cullins mit NEDD8 aktiviert, während DCN1 Cullinneddylierung und daher auch Ubiquitinligaseaktivität reguliert (Kurz, Chou et al. 2008). Unsere Resultate deuten darauf hin, dass *NtDCN*1 für Entwicklungstransitionen während der Pollenentwicklung und Embryogenese in Pflanzen verantwortlich ist (Hosp 2009).

Basierend auf diesen Daten, wollten wir die Funktion von einem *Arabidopsis thaliana DCN*1Homolog (At3g12760) analysieren; daher wurden Samen einer T-DNA Insertionslinie (ID 814C11) von GABI Kat bestellt, welche eine Insertion in dem zehnten Exon des gewünschten Gens enthält. Anfängliche Versuche mit der T2 Generation von 814C11 bestätigten die Position der Insertion in *AtDCN*1, aber wie auch von GABI Kat angemerkt, wiesen sie auf eine zweite Insertion im Mutanten Genom hin. Um die funktionelle Rolle von *DCN*1 in der Modelpflanze *Arabidopsis thaliana* zu erforschen, musste eine der zwei ermittelten T-DNA Insertionen mittels Rückkreuzungen zu einem Wildtyp segregiert werden. Nach intensiver Forschung auf molekularer Basis mit einer Menge von rückgekreuzten Pflanzen fanden wir heraus, dass zwei Insertionen in *AtDCN*1 lokalisiert sind, welche RB zu RB zueinander liegen; daher ist eine Segregation unmöglich, aber auch nicht notwendig, weil beide Insertionen das gewünschten Gen disruptieren. Durch PCR auf genomischer DNA Ebene und Expressionsanalysen von *AtDCN*1 konnten wir eine positive, rückgekreuzte Pflanze (BX3a 814C11/24-6-10 x wt A5) identifizieren, welche homozygot für die Mutation ist.

3. ABSTRACT

Plant proteomes show remarkable plasticity which enables them to react to environmental challenges and to accomplish developmental transitions (Hosp 2009). Our group have isolated and analyzed a tobacco (*Nicotiana tabacum*) gene encoding DEFECTIVE IN CULLIN NEDDYLATION 1 (*DCN*1), a homolog of yeast (*Saccharomyces cerevisiae*) and worm (*Caenorhabditis elegans*) DCN1 that binds ubiquitin and RUB/NEDD8, and associates with cullins and an E2-conjugating enzyme for NEDD8 (Hosp 2009). Cullin based ubiquitin ligases are activated through modification of the cullin subunit with NEDD8, while DCN1 regulates cullin neddylation and thus ubiquitin ligase activity (Kurz, Chou et al. 2008). Our results indicate that *NtDCN*1 is required for developmental transitions during pollen development and embryogenesis in plants (Hosp 2009).

Based on these data we wanted to analyse the function of an *Arabidopsis thaliana DCN*1 homolog (At3g12760); therefore seeds of a T-DNA insertion line (ID 814C11) were ordered from GABI Kat, which were shown to have an insertion within the 10th exon of the desired gene. Initial research on the T2 generation of 814C11 plants approved the insertion to be located within *AtDCN*1, but as GABI Kat mentioned our results also indicated for a second insertion within the mutant genome. To investigate the functional role of *DCN*1 in the model plant *Arabidopsis thaliana*, one of the two determined insertions have to be segregated out via backcrossing to a wild type background. After intensive research on molecular level on backcrossed plants (BX) we found out that two T-DNA insertions are located RB to RB to each other, hence an out-segregation of the second insertion is impossible, but also not sufficient for further molecular analysis because both insertions are disrupting the same gene. Furthermore PCR on genomic DNA level as well as expression analysis of *AtDCN*1 lead to the identification of one positive backcrossed plant (BX3a 814C11/24-6-10 x wt A5) which is homozygous for the mutation.

4. INDEX of CONTENTS

5. INTRODUCTION

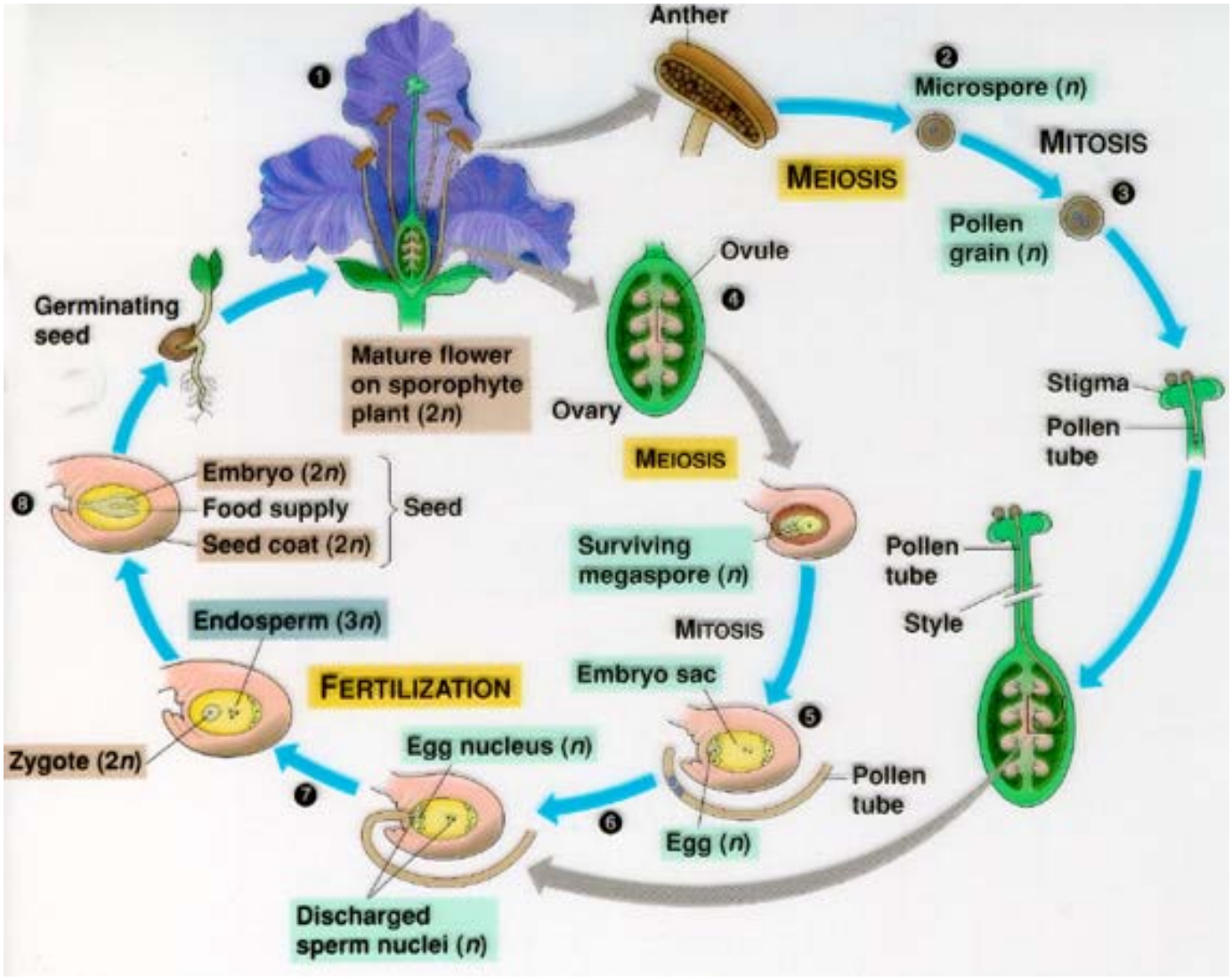
5.1. Alternation of generations in plants

Higher plants are confined by their habitat of germination and meet this challenge by remarkable proteomic plasticity (Dreher and Callis 2007; Stone and Callis 2007). They undergo various phase changes and reprogramming during development and stress adaptation, and some of these have been shown to be accompanied by large-scale epigenetic and gene expression changes (Poethig 2003; Arnholdt-Schmitt 2004; Bruce and Pickett 2007; Lopez-Maury, Marguerat et al. 2008; Mazzucotelli, Ribet et al. 2008).

The most fundamental transition in the life cycle of higher plants is the alternation of generations (McCormick 2004;Yadegari and Drews 2004). Early in their evolution, plants acquired this life cycle that alternates between a multicellular haploid organism, the gametophyte, and a multicellular diploid organism, the sporophyte (Fig. 1) (Yadegari and Drews 2004). During the angiosperm life cycle, the sporophyte produces two types of spores, microspores and megaspores, that give rise to male gametophytes and female gametophytes, respectively (Yadegari and Drews 2004; Jakobsen, Poulsen et al. 2005). Both gametophytes are multicellular structures and are essential for the reproductive process (Yadegari and Drews 2004; Jakobsen, Poulsen et al. 2005).

The female gametophyte, also referred as the embryo sac or megagametophyte, develops within the ovule, which is found within the carpel's ovary, over two phases referred to as megasporogenesis and megagametogenesis (Yadegari and Drews 2004). During megasporogenesis, the diploid megaspore mother cell undergoes two meiotic divisions, resulting in four one-nucleated megaspores, while three megaspores subsequently undergo cell death (Yadegari and Drews 2004). During megagametogenesis, the functional megaspore undergoes one or more rounds of mitosis, giving rise to the mature female gametophyte (Yadegari and Drews 2004). The most common female gametophyte form consists of seven cells and four different cell types: three antipodal cells, two synergid cells, one egg cell, and one central cell (Maheshwari and Johri 1950).

The male gametophyte, also referred as the pollen grain or microgametophyte, develops inside the anther, a specialized structure of the flower (McCormick 1993; Jakobsen, Poulsen et al. 2005).



[1]

Fig. 1 Life cycle of higher plants

Male gametogenesis begins with meiotic divisions of a sporogenous initial cells, also called pollen mother cell (PMC) to form a tetrade of haploid spores (McCormick 1993; McCormick 2004; Jakobsen, Poulsen et al. 2005). These microspores are embedded in callose, and release of the individual cells of the tetrade requires the action of an enzyme (callase) produced by the tapetum layer of the anther (Stieglitz 1977; McCormick 1993; Rhee and Somerville 1998). The uninucleate microspores enlarge and then each undergo an asymmetric mitotic division, resulting in a bicellular pollen grain containing a larger vegetative cell and a smaller generative cell that is enclosed entirely within the vegetative cell (McCormick 1993; McCormick 2004). The two cells of the bicellular pollen grain have strikingly different fates, while the larger vegetative cell does not divide again but eventually will form the pollen tube (McCormick 2004). The smaller generative cell undergoes a second mitotic division to form

[1] http://fig.cox.miami.edu/Faculty/Dana/angiolifecycle.jpg

two sperm cells (McCormick 1993; McCormick 2004). Soon after the mature pollen is transferred from the anther to the stigma, the male gametophyte forms a pollen tube that grows via a tip-growth process through the carpel's sporophytic tissue to reach the female gametophyte (Yadegari and Drews 2004). Double fertilization occurs when the two sperm cells migrate to the egg and central cell and their plasma membranes fuse with the respective target cell to transport the sperm nuclei for karyogamy (van Went 1984; Russell 1992; Russell 1996). The fusion of egg and sperm gives rise to the zygote, which is the beginning of diploid sporophyte generation, thereby completing the life cycle (Gifford 1989).

5.2. Microspore embryogenesis

Isolated microspore cultures have the remarkable ability of resembling the alternation of generations in the life cycle of flowering plants, i.e. the change between the diploid sporophytic and the haploid gametophytic generation (Heberle-Bors 1989). Although the natural destination of microspore development is to differentiate into mature pollen and accomplish fertilization, microspores or young pollen grains can either differentiate into mature pollen (the male gametophytes) by culture in a rich medium, or divide repeatedly and develop into embryos (sporophytes) after a stress treatment (Touraev 1997). This process is called androgenesis or microspore embryogenesis and is widely used in plant breeding programmes to generate homozygous lines for breeding purposes (Hosp 2007). The majority of information regarding the genetic and molecular control of the developmental switch from gametophytic to sporophytic development has been garnered from four intensely studied (crop) plants comprising two dicotyledonous species, rapeseed (*Brassica napus*) and tobacco (*Nicotiana tabacum*), and two monocotyledonous species, wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Hosp et al. 2007). Although *Arabidopsis thaliana* has been the model of choice for most plant molecular-genetic studies, microspore embryogenesis has not yet been attained despite much (unpublished) effort (Hosp et al. 2007).

In *Nicotiana tabacum*, sucrose and nitrogen starvation of immature isolated young bicellular pollen grains induces the formation of embryogenic pollen, which, after transfer to a simple, sucrose and nitrogen-containing medium, divides repeatedly and produces large numbers of embryos (Kyo 1986; Garrido 1991). A heat-shock treatment is not effective at this stage of development but can induce embryogenesis at an earlier stage when unicellular microspores are used (Touraev 1996). A combination of starvation and heat stress induces

embryogenesis in nearly all of the living microspores while under non-stress conditions in a rich medium the microspores develop into mature fertile pollen (Benito Moreno R. M. 1988; Touraev and Heberle-Bors 1999). The chromosome number of microspore-derived haploid seedlings can easily be doubled by using chromosome doubling agents like colchicin, giving rise to fully fertile homozygous double haploid plants, which are used either directly as varieties, or as inbred lines in F1 hybrid production (Kasha 2003; Hosp, Tashpulatov et al. 2007).

Microspore embryogenesis has been proven to be an essential and indispensable tool for modern plant breeding and has developed into a valuable system to answer questions concerning plant cell totipotency and embryo development (Boutilier 2004; Forster 2005).

5.3. The *DCN*1 homolog *NtSM*10 is involved in tobacco microspore embryogenesis and pollen development

After exposure to a variety of stress treatments such as heat and/ or starvation, isolated and cultured plant microspores can be diverted from their normal gametophytic pathway towards sporophytic development, with the formation of haploid embryos and ultimately double haploid plants (Hosp 2007). As our group was interested in the identification of genes involved in microspore embryogenesis, suppression subtractive hybridisation (SSH) was applied for the identification of differentially expressed genes in stressed and non-stressed microspores, which led among others to the isolation of *NtSM*10 (*Nicotiana tabacum* Stressed Microspore; further renamed as *NtDCN*1), a gene strongly up-regulated in stressed, embryogenic tobacco microspores (Hosp 2006). *NtDCN*1 (Defective in Cullin Neddylation), a homolog of yeast (*Sachharomyces cerevisiae*) and worm (*Caenorhabditis elegans*) *DCN*1, shows a full-length cDNA of 1155 bp, encodes a 30 kDa protein comprising 259 amino acids and contains UBA, PONY and EF-Hand domains. DCN1 binds ubiquitin and RUB/NEDD8, which is closely related to ubiquitin, and associates with cullins and an E2-conjugating enzyme for NEDD8 (Hosp 2009). Cullin-based E3 ubiquitin ligases are activated through modification of the cullin subunit with the ubiquitin-like protein NEDD8, while *DCN*1 has been characterized as an E3 ligase for cullin rubylation/neddylation, thus regulating ubiquitin ligase activity (Kurz, Chou et al. 2008; Hosp 2009).

To analyze the expression pattern of the *NtDCN*1 gene, promoter:*GUS* fusion plants were used (Hosp 2009). Histochemical analysis of transgenic tobacco lines harbouring the *DCN*1pro:*GUS* construct revealed that *NtDCN*1 is expressed throughout the plant body, and that expression is differentially regulated during pollen development and in reprogrammed cultured microspores (Hosp 2009). Reverse Northern, Northern and *in situ* hybridization analysis confirmed that *NtDCN*1 is highly elevated in stressed tobacco microspores and microspore-derived embryos. Knock-down of *NtDCN*1 by RNAi resulted in an arrest of pollen formation after the first pollen mitosis, in a block of zygotic embryogenesis around the globular stage, and moreover impeded the stress-triggered reprogramming of cultured microspores from their intrinsic gametophytic mode of development to an embryogenic state (Hosp 2009). RNAi lines also revealed changes in cullin neddylation compared to wild type protein pattern checked by anti-RUB/NEDD8 Western blots. By retransforming RNAi lines with a transcriptional gene silencing (TGS) construct directed against the RNAi-driving promoter (Sijen, Vijn et al. 2001), our group was able to demonstrate restoration of the *NtDCN*1 phenotypes. To find out more about the function of DCN1 during pollen development and reprogramming of microspores, transgenic plants were created that harboured the *DCN*1 full-length cDNA under control of a DC3 promoter, shown to be highly active during male gametophyte development and microspore embryogenesis (Wilde 1988; Touraev 1995). Over-expression of *NtDCN*1 accelerated pollen tube growth and promoted the formation of embryogenic microspores by reducing the duration of the stress treatment required to reprogram microspores into sporophytic development.

All results together indicate that *NtDCN*1 is required for developmental transitions during pollen development and embryogenesis in plants (Hosp 2009).

5.4. DCN1 structure is evolutionary conserved among all eucaryotes

A few years ago Kurz, Ozlu et al reported the identification and analysis of a widely conserved protein that is required for cullin rubylation/neddylation in the nematode *Caenorhabditis elegans* and the yeast *Saccharomyces cerevisiae* (Kurz, Ozlu et al. 2005). The Dcn1 (defective in cullin neddylation) full-length protein has 269 amino acids and its structure revealed an ordered core domain encompassing residues 66-269 (Yang, Zhou et al. 2007). X-ray crystal structure analysis established that yeast Dcn1 contains an N-terminal UBIQUITIN-ASSOCIATED (UBA) domain, followed by a disordered linker and a large C-

terminal POTENTIATING NEDDYLATION (PONY) domain of unique architecture, formerly known as DUF298 (Kurz, Chou et al. 2008) (Fig. 2). Both domains form an elongated structure composed of eleven α helices, while the N-terminal domain (residues 66-158) encompasses helices α1 - α5 and a C-terminal domain (residues 159-269) containing helices α6 - α11 (Yang, Zhou et al. 2007; Kurz, Chou et al. 2008).

Fig. 2 DCN1 protein features

UBA domains have been found in a variety of proteins linked to the ubiquitin pathway (Hofmann and Bucher 1996), where they have been shown to interact directly with ubiquitin (Meyer, Wang et al. 2002). The UBA domain of yeast Dcn1 displays the three-helix canonical architecture shared by other UBA domains (Hofmann and Bucher 1996; Hicke, Schubert et al. 2005) and was also shown to specifically bind ubiquitin (Kurz, Chou et al. 2008). To further investigate the functional relevance of the UBA domain, Kurz, Chou et al. constructed a mutant form of Dcn1 lacking the first 69 amino acids, resulting in a truncated protein that entirely consists of the PONY domain (Kurz, Chou et al. 2008). This mutant was fully capable of promoting cullin neddylation, as expression of this mutant in yeast lacking endogenous Dcn1 restored neddylation of the yeast cullin-1 homolog Cdc53, suggesting that ubiquitin binding is not essential for Dcn1-dependent cullin neddylation *in vivo* (Kurz, Chou et al. 2008).

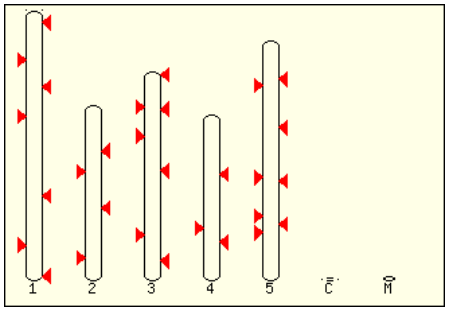


Fig. 3 Ubiquitin-associated domain

This figure shows the location of genes in the model plant *Arabidopsis thaliana*, containing an ubiquitin-associated domain, indicated with red arrowheads.
http://atensembl.arabidopsis.info/Arabidopsis_thaliana_TAIR/domainview?domainentry=IPR000449

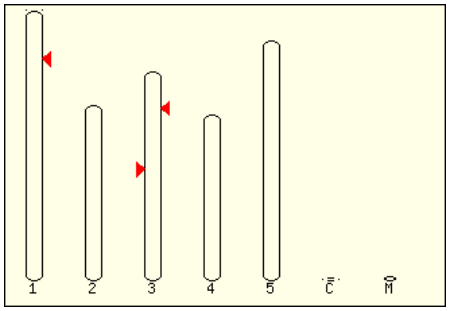


Fig. 4 PONY domain

This figure shows the location of just three genes (At1g15860, At3g12760, At3g28970) in the model plant *Arabidopsis thaliana*, containing a PONY domain, indicated with red arrowheads.
http://atensembl.arabidopsis.info/Arabidopsis_thaliana_TAIR/domainview?domainentry=IPR005176

In contrast to its interaction with ubiquitin, the DCN1 UBA-like domain by itself only weakly interact with Nedd8, suggesting that the UBA-like domain is specific for ubiquitin, and that Nedd8 binding is probably mediated by a different part of DCN1 (Kurz, Ozlu et al. 2005). The PONY domain consists of a superhelical arrangement of α helices (Kurz, Chou et al. 2008) and revealed the presence of two EF-Hand-like elements, which typically function to bind calcium (Kawasaki, Nakayama et al. 1998). However, both EF-Hands lack some of the conserved residues that enable calcium binding (Kurz, Chou et al. 2008). The PONY domain is evolutionarily conserved, with a single gene encoding family members in *C. elegans*, *S. cerevisiae* and *Schizosaccharomyces pombe*, and a limited number of homologs in other eucaryotes (Kurz, Ozlu et al. 2005). This carboxy-terminal domain was formerly known as DUF298 domain of unknown function, is unique to Dcn1 and is present in all recognizable Dcn1 homologs (Kurz, Ozlu et al. 2005). To analyze the function of this domain, Kurz, Chou et al. constructed mutants with single point mutations in the conserved surface (D226, A253, D259; hereafter referred as the DAD patch) of the PONY domain (Kurz, Chou et al. 2008). In contrast to wild type Dcn1, all three single point mutations displayed a small but significant reduction in cullin neddylation; while double and triple mutant combinations caused a complete loss in the ability to promote cullin-1 homolog Cdc53 neddylation (Kurz, Chou et al. 2008). These data suggested that the molecular surface comprising the DAD patch is functionally important for the ability of Dcn1 to promote neddylation *in vivo*, which suggested that all critical and essential neddylation functions are contained within the PONY domain (Kurz, Chou et al. 2008). Furthermore it was shown by Kurz, Ozlu et al that DCN1 binds directly and tightly to Cul1 family members through the DAD patch, and that the interaction between the two proteins is important for neddylation (Kurz, Chou et al. 2008). DCN1 also provides a surface for interaction with a NEDD8 E2 that overlaps with a NEDD8 E1-binding site.

DCN1/ Dcn1p acts as a scaffold protein in a RUB/NEDD8 complex in association with Rbx1 as shown in yeast (Yang, Zhou et al. 2007). Recently it was shown by Kurz, Ozlu et al that Dcn1 is necessary and sufficient for cullin rubylation/ neddylation in a purified system (Kurz, Chou et al. 2008), while covalent modification of cullins by RUB/NEDD8 and removal of RUB/NEDD8 by the COP9 signalosome positively regulate ubiquitin E3 ligase activity, thus enhancing ubiquitylation of substrates (Schwechheimer and Calderon Villalobos 2004). The mouse DCN1 homolog known as RP42 has been associated with autism and shows developmentally regulated expression in proliferating neuroblasts (Mas, Bourgeois et al. 2000) and testis (Pourcel, Jaubert et al. 2000). In *C. elegans,* loss of DCN1 causes

embryonic arrest due to loss of CUL-3 activity, while in budding yeast, a DCN1 null mutant is viable, consistent with the observation that RUB/NEDD8 is not essential in budding yeast (Kurz, Ozlu et al. 2005). The human DCN1 homolog SQUAMOUS CELL CARCINOMA-RELATED ONCOPROTEIN (SSCRO) is a proto-oncogene with possible function in hedgehog signalling (Sarkaria, P et al. 2006) and has recently been described as a component of the E3 neddylation complex, like in yeast DCN1 (Kim, Bommelje et al. 2008), confirming its important and conserved function.

Since most components of the ubiquitin-proteasome pathway are conserved throughout the eucaryotic kingdoms (Hellmann and Estelle 2002), the existence of a DCN1 homolog in plant genomes comes as no surprise. The *Arabidopsis thaliana* genome contains more genes encoding ubiquitin conjugases and ubiquitin ligases compared to other model organisms, like *S. cervisiae* or *C. elegans*, suggesting the importance of ubiquitin-mediated protein degradation during plant development (Bachmair, Novatchkova et al. 2001).

Fig. 5 Comparison of secondary structure prediction and domain structure of DCN1 homologs and DCN1 like genes in *Arabidopsis thaliana*.

Amino acids conserved among all DCN1 and DCN1-like genes are highlighted in green; among three proteins – in red, and between two proteins – in blue. Regions with a high probability of α-helix formation are indicated with H,UBA domains are marked with a grey box, PONY domains in a yellow box, and EF-hand domains EF1, EF2 in orange blocks.

The closest homolog of *NtDCN*1 in *Arabidopsis thaliana* was found to be an unknown protein (At3g12760) with 89% similarity (78% identity and similar domain architecture that includes the UBA and the PONY domain) (Hosp 2009). At3g12760 (*AtDCN*1) encodes a 29 kDa protein comprising 250 amino acids (http://www.arabidopsis.org/servlets/TairObject?id=37952&type=locus). Other close relatives were found to be DCN1-like proteins which share 45% (At3g28970, *AAR*3) and 32% identity (At1g15860). The gene expression maps of *AtDCN*1 and *DCN*1-like genes in Arabidopsis development are shown in Fig. 6-8. The alignment in Fig. 5 shows the secondary structure prediction and domain structure of the tobacco and Arabidopsis *DCN*1 homologs and *AtDCN*1-like genes, while At1g15860 and *AAR*3 (anti auxin resistant 3) miss the N-terminal UBA domain. *AAR*3 has recently been identified through an auxin-resistant mutant screen in Arabidopsis, was shown to regulate responses to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) in roots, and may also be involved in the regulation of the SCF-dependent protein degradation system (Biswas, Ooura et al. 2007). Auxin is important in many aspects of plant development, such as embryogenesis (Jenik and Barton 2005) as well as anther and pollen development (Cecchetti, Altamura et al. 2008). However, further investigations are required to elucidate the diverse and/or redundant roles of *DCN*1 and *DCN*1-like genes in development and stress response in general and in auxin-dependent developmental processes (Hosp 2009).

2

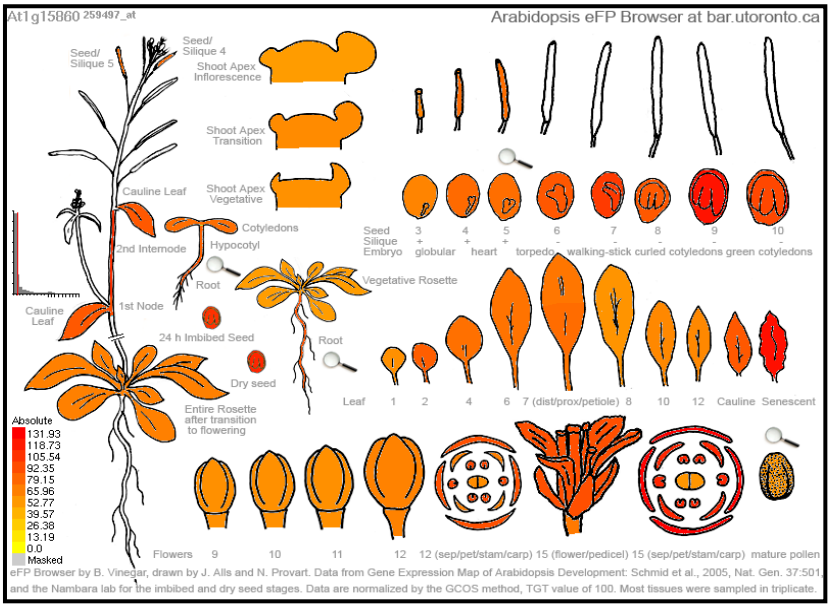


Fig. 6 At1g15860

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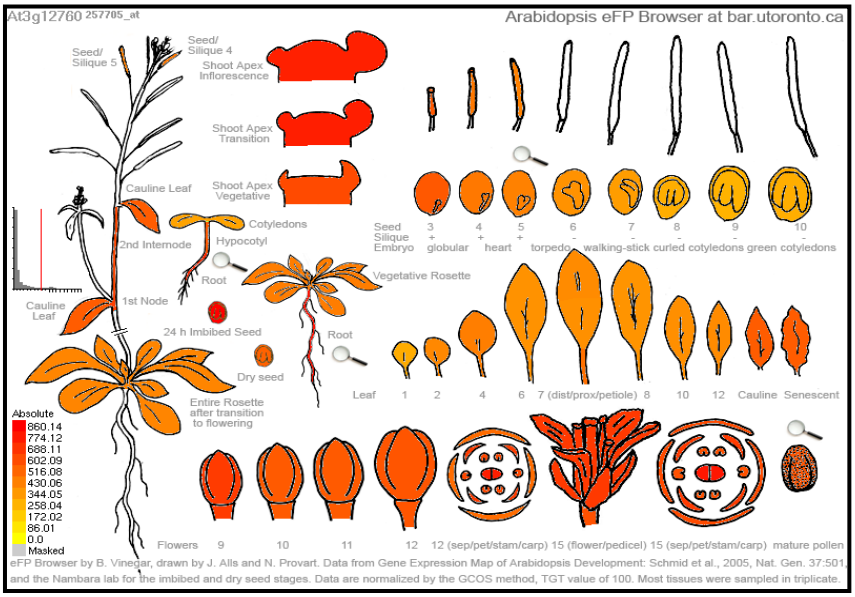

Fig. 7 At3g12760

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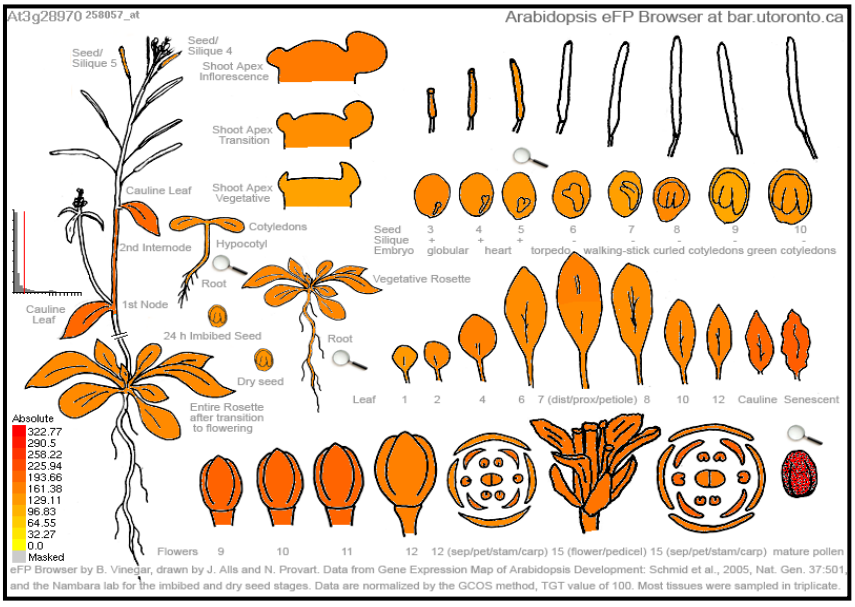

Fig. 8 At3g28970

5.5. Ubiquitin-mediated protein degradation and the role of *DCN*1 in Cullin Rubylation/Neddylation

Plants as sessile organisms, rely on proteomic plasticity to adapt to periods of developmental change, to endure environmental conditions, and to respond to biotic and abiotic stresses

[3] www.genevestigator.ethz.ch
[4] www.genevestigator.ethz.ch

(Dreher and Callis 2007). Post-translational control of protein turnover by ubiquitination and degradation by the 26S proteasome is a highly regulated process essential for all eucaryotes (Stuttmann, Lechner et al. 2009). The pathway consists of three protein complexes, called ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) (Dharmasiri, Dharmasiri et al. 2003), and of a tightly controlled cascade of those enzymes, the activities of which result in covalent transfer of a small protein, ubiquitin, to specific substrates and the subsequent degradation of the ubiquitinated substrate proteins by the 26S proteasome (Hotton and Callis 2008). It is predicted that there are more than 1000 ubiquitin E3 ligases in plants (Schwechheimer and Calderon Villalobos 2004), which regulate many developmental and physiological responses such as hormone signalling, cell division, floral development and maintenance of circadian rhythm (Moon, Parry et al. 2004; Smalle and Vierstra 2004).

The largest class of E3s are the multisubunit cullin-RING ligases (CRLs), each of which contains a cullin protein, a RING protein and a substrate receptor protein (Petroski and Deshaies 2005). Cullins are post-translationally modified by the covalent attachment of RUB/NEDD8 (Pan, Kentsis et al. 2004), an 8 kDA protein that is closely related to ubiquitin (Schwechheimer and Calderon Villalobos 2004), in a process called cullin rubylation/neddylation. RUB/NEDD8 is highly conserved in most eucaryotes (plants, slime molds, fungi and animals) (Kumar, Tomooka et al. 1992; Rao-Naik, delaCruz et al. 1998; Burroughs, Balaji et al. 2007)) - also known as RUB1 in *S. cerevisiae* – but unlike ubiquitin, however, NEDD8 does not form chains and does not target proteins for degradation (Hochstrasser 2000). Regardless, the proteins that mediate neddylation are closely related to E1 and E2 enzymes of the ubiquitylation pathway (Kurz, Chou et al. 2008). Similar to ubiquitin, NEDD8 is attached to its substrate by an isopeptide linkage between its carboxy-terminal gylcine (Gly) 76 and a lysine of the target protein (Rabut and Peter 2008).

In vitro biochemical studies focussing on the role of cullin rubylation/neddylation suggest; that the catalytic activity of an assembled CRL is increased when modified by RUB/NEDD8, thus enhancing ubiquitylation of substrates (Kawakami, Chiba et al. 2001; Wu, Chen et al. 2002; Morimoto, Nishida et al. 2003).

Arabidopsis expresses four functionally relevant cullins giving rise to three distinct classes of CRLs (Stuttmann, Lechner et al. 2009). The best characterized CRLs are the SCF complexes, also required for degradation of key regulatory proteins involved in cell cycle progression, development, and signal transduction (Patton, Willems et al. 1998; Seol, Feldman et al. 1999; Kipreos, Gohel et al. 2000). SCF complexes are multi-protein complexes

and contain four subunits: Skp1; CUL1; an F-box protein; and the RING H2 finger protein Rbx1/Roc1/Hrt1(Kurz, Ozlu et al. 2005; Petroski and Deshaies 2005). Substrate specifity of those complexes is conferred by F-Box proteins which associate with CUL1 (acts as a rigid backbone) via SKP1 adaptor protein (Stuttmann, Lechner et al. 2009), while Rbx1 binds to the C-terminus of CUL1 and facilitates the recruitment of E2 to the complex (Bosu and Kipreos 2008). Cullins interact tightly with the RING-domain protein Rbx1 (Ohta, Michel et al. 1999), forming the catalytic core of the SCF-type E3 ubiquitin ligase which recruits charged ubiquitin E2s into the complex and catalyses the ubiquitination of cullin substrates (Seol, Feldman et al. 1999) (Fig. 9). The probably best-characterized SCFs are the SCFTIR1/AFB complexes, which function as receptors for the plant phytohormone auxin (Dharmasiri, Dharmasiri et al. 2005; Kepinski and Leyser 2005). In Arabidopsis, SCFs (in detail F-Box proteins) have been implicated in senescence (Woo, Chung et al. 2001), apical dominance (Stirnberg, van De Sande et al. 2002), circadian rhythm (Nelson, Lasswell et al. 2000; Somers, Schultz et al. 2000), flower and meristem development (Ingram, Doyle et al. 1997; Samach, Klenz et al. 1999) and phytochrome A signaling (Dieterle, Zhou et al. 2001).

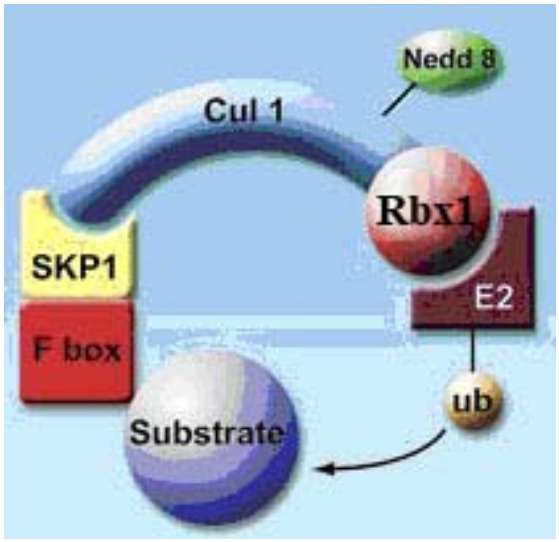

Fig. 9 SCF complex
http://stke.sciencemag.org/content/sigtrans/vol2006/issue335/images/large/3352006pe21F1.jpeg

RUB/NEDD8 modification is antagonized by the COP9 signalosome , an evolutionary conserved eight-subunit complex (Stuttmann, Lechner et al. 2009), originally identified in Arabidopsis (Wei and Deng 1992), which is essential in most eucaryotes and cleaves RUB/NEDD8 from cullins (Stuttmann, Lechner et al. 2009). Biochemically, CSN is associated with three activities, phosphorylation, deneddylation, and deubiquitination, with the latter two activities directly regulating CRLs (Cope and Deshaies 2003; Wolf, Zhou et al.

2003). Since RUB/NEDD8 conjugation promotes E3 ligase activity, RUB/NEDD8 cleavage by the CSN is expected to act as a negative regulator of CRL activity (Stuttmann, Lechner et al. 2009). Though, genetic evidence in several organisms has revealed that the CSN promotes cullin activity, indicating that cycles of neddylation and deneddylation are required for correct cullin function *in vivo* (Cope and Deshaies 2003; Pintard and Peter 2003; Bosu and Kipreos 2008). This apparent paradox was first reconciled in fission yeast, as CSN was shown to protect CRL substrate adaptors from autocatalytic degradation through both its derubylation/deneddylation and a CSN-associated deubiquitination enzyme (Wee, Geyer et al. 2005). In Arabidopsis, the characterization of *csn* mutants has been limited by their early seedling lethality (Stuttmann, Lechner et al. 2009).

In general the RUB/NEDD8 conjugation pathway is essential for viability in mice (Tateishi, Omata et al. 2001), *C. elegans* (Jones and Candido 2000), and *Arabidopsis thaliana* (Bostick, Lochhead et al. 2004), while in budding yeast, a DCN1 null mutant is viable, consistent with the observation that RUB/NEDD8 is not essential in budding yeast (Kurz, Ozlu et al. 2005).

As Dcn1 is involved in microspore embryogenesis and pollen development (Hosp 2009) and was shown to function as a scaffold-type E3 ligase for cullin neddylation (Kurz, Chou et al. 2008), thus enhancing ubiquitylation of substrates (Morimoto, Nishida et al. 2003), it would be interesting to investigate the role of ubiquitin-mediated protein degradation during microspore reprogramming after stress treatment. Stress might not only block the execution of predetermined gametophytic program, but may also induce an autophagic re-utilization of gametophyte-specific products, thus converting microspores to non-specialized, totipotent cells (Hosp, Tashpulatov et al. 2007). It has become widely accepted that the degradation of cellular components and proteins is a prerequisite for developmental re-programming in many organism; and that this re-programming is mediated on one hand by the 26S proteasome and on the other hand through autophagy thus lysosomal recycling (Hosp 2007). At some stage in androgenic development proteins are assumed to be recycled and selectively destroyed in favour of synthesis of new proteins that are able to better serve the novel conditions of embryogenic induction, while a number of protease and ubiquitin-interacting genes isolated in independent studies support this assumption (Hosp 2007).

5.6. T-DNA Insertion lines

Reverse genetics is a strategy to determine a particular gene's function by studying the phenotypes of individuals with alterations in the gene of interest (Sessions, Burke et al. 2002). In contrast to forward genetics, reverse genetics begins with a mutant gene sequence and asks the question "What is the resulting change in phenotype?" (Krysan, Young et al. 1999). Nowadays, many methods are known to knock-out or knock-down the expression of any gene of interest, providing powerful systems to study the physiological function of a protein.

Insertional mutagenesis is an alternative means of disrupting gene function and is based on the insertion of foreign DNA into the gene of interest (Krysan, Young et al. 1999). Since Marc Van Montague and Jeff Schell discovered the gene transfer mechanism between *Agrobacterium tumefaciens* and plants, and since protocols for Agrobacterium-mediated transformation were developed, this gene transfer mechanism is routinely used for the genetic modification of a wide range of plant species (Schell and Van Montagu 1977; Marcel J.A. de Groot 1998). *Agrobacterium tumefaciens* transfers part of its Ti plasmid, the T-DNA delimited by two 24-bp border repeats (referred as left border (LB) and right border (RB)), to plant cells where it becomes randomly integrated into the host genome by the plant's illegitimate recombination apparatus (Matsumoto, Ito et al. 1990; Gheysen, Villarroel et al. 1991; Mayerhofer, Koncz-Kalman et al. 1991; Marcel J.A. de Groot 1998). T-DNA insertions are stable because of their integration into the host's genome, not only disrupt the expression of the gene into which it is inserted but also acts as a selection marker for subsequent identification of mutant plants (Krysan, Young et al. 1999), making such knock-out mutants a frequently used powerful system for study the function of a gene (Wormit, Trentmann et al. 2006). Nowadays whole collections of T-DNA mutagenized *Arabidopsis thaliana* lines are established and seeds of such insertion lines are commercially available from institutes such as GABI-Kat (Genomanalyse im biologischen System Pflanze) or SALK institute for biological studies. For further mutant characterization seeds are planted, grown to maturity and plant material is analysed on molecular level via e.g. PCR or different blotting methods. If the mutant plants are shown to have more insertions within their genome the additional insertions can be segregated out via backcrossing to a wild type background. Homozygous lines with one insertion within the plants genome show a complete (100%) knock-out of the expression of the disrupted gene, hence providing a perfect basis for further functional

analysis, while mutations that are homozygous lethal can be maintained in the population in the form of heterozygous plants (Krysan, Young et al. 1999).
Another method which is used for functional genomics is to knock-down the expression of an interesting gene by e.g. antisense RNA or RNA interference (RNAi). Both work in a similar sequence specific manners and lead to a transient gene silencing due to its poor stability. Antisense oligonucleotides as well as specific inheritable RNAi are used for sequence-specific silencing of gene function, by hybridization to the complementary mRNA and subsequent degradation by either RNAse H (antisense RNA) or an RNA-induced silencing complex (RNAi). An advantage of targeted gene knock-down is that it can be used in all plants which can be transformed with an RNAi construct, while commercially available knock-out T-DNA insertions lines are limited to a number of genes and plant species. A drawback of the RNAi approach is that routine complementation of observed phenotypes as is done in *Arabidopsis thaliana* knock-out mutants has not been established, making T-DNA insertions a popular system to study gene function.

5.7. Aim of the project

The aim of this project is the molecular characterization and analysis of a putative *AtDCN*1 knock-out line 814C11, therefore seeds of a putative *AtDCN*1 T-DNA insertion line were ordered from GABI Kat, characterized on molecular level and checked for altered phenotypes.

6. MATERIALS and METHODS

6.1. Sterilization, propagation and collection of seeds

For the sterilization seeds of T-DNA insertion lines were transferred into an Eppendorf tube and washed once with 200-300 µL 70% ethanol under a laminar flow. The supernatant was discarded after short centrifugation. Eppendorf tube with sterilized seeds was kept open in the laminar flow to allow evaporation of residual ethanol, which might have a negative effect on germination. As a control, the whole procedure of sterilizing until seed germination was always performed with wild type Arabidopsis cv. col-O seeds in parallel.

Seeds were germinated in agarose solidified MS medium. Petri dishes of approximately 10 cm containing medium MS have been prepared by pouring autoclaved liquid medium MS onto the surface of Petri dishes and plates were solidified under the laminar flow by keeping lids open for some time.

Sterilised seeds in Eppendorf tubes were resuspended with 300-500 µL ddH_2O^a , the seeds were pipetted on a sterile filter paper and transferred carefully onto solid MS plates using a small, thin forceps. The seeds were placed in a small distance to each other to facilitate the later transfer of the seedlings into soil. To avoid any contamination, the MS plates were sealed with Parafilm and for synchronisation of germination, MS plates were incubated for two days at 4°C following by culture for approximately two weeks in the climate chamber under optimal controlled condition for germination (Light: 16 hours (8 hours dark); temperature: 24°C (+/- 2°C); humidity: 50% (+/- 5%)).

Seedling with two to three cotyledone pairs were transferred into pots with *Arabidopsis thaliana* soil mixture (five parts N_3 Humin Substrate N3, one part white Granuperl perlite). To provide perfect conditions, growing seedlings were maintained under a plastic cover for the first two days in the greenhouse (temperature: 19 °C (+/- 7°C); humidity: 52% (+/- 5%)).

From the third day on the plants were watered every second to third day and as the seedlings were grown to a certain height, they were stabilized with a bamboo stick.

Approximately four weeks after planting, the watering was stopped. The siliques were collected as they become yellow, in a way like placing a collection baggy over the plant and pulling off the siliques from the stem into the baggy. Obtained seeds were dried for approximately two weeks at room temperature to make them ready for further propagation if necessary.

6.2. Agarose gel electrophoresis

Gel electrophoresis is a technique used to analyze the size and amount of DNA fragments in comparison to a length marker with fragments of known size (e.g. Lambda/ pst). Electrophoresis uses electric fields (usually 65-100 V adjustably on a power supply (EC 105; E-C Apparatus Corporation)) to pull negatively charged DNA molecules through an agarose gel, separating DNA fragments by their size (range 150-15000 bp), as shorter fragments are able to move faster than longer ones.

For preparation of the gel, the agarose was weight into a flask, filled up with 1x TAE buffer and heated in a microwave to melt the agarose. As the solution has cooled down to approximately 55-60°C, 1-1,5 µL ethidium bromide (EtBr) were added before the still liquid gel was poured into foreseen trays equipped with a comp. EtBr plays an important role in gel electrophoresis, as firstly it unfolds DNA and enables the linear size of DNA fragments to be analyzed. Secondly, EtBr intercalates with DNA, while this combination fluoresces when exposed to UV-light, therefore enabling the DNA bands to be seen on the gel.

DNA samples to be analyzed mixed with 6 x TAE loading dye were loaded on the agarose gel using 1x TAE as a running buffer. After separation of the DNA fragments by applying a proper voltage, the gel was examined and photographed under UV-light using a Transiluminator.

6.3. Quantification of nucleic acids

DNA concentrations were determined by photometric measurement at 340 nm using an 1:1000 dilution (diluted in ddH_2O^a). The quantification of RNA was measured at 260 nm, using a 5:1000 dilution (diluted in ddH_2O^a supplemented with DEPC), while protein concentrations were determined at 595 nm (2:1000 dilution in ddH_2O^a and Bradford reagent). Either, cuvettes out of quartz were used for nucleic acid quantification, or out of plastic for protein quantification.

6.4. Tissue Lyser

The Tissue Lyser system was used to facilitate the grinding process of the plant material for further DNA or RNA isolation. One to two beads (Precellys-Stahl-Kit 2,8 mm) and 100-200 mg plant material were transferred to an Eppendorf tube and frozen in $N_{2\ liquid}$. Those samples were packed into two pre-cooled cooling units in a way that the samples were balanced followed by fixing those units in foreseen notches on the apparatus. As the Tissue Lyser program (one to two minutes, 30 seconds frequency) has finished any protocol for further isolation was followed immediately to prevent degradation of the pulverized plant material.

6.5. DNA isolation/ CTAB method

For DNA isolation via CTAB method leaves from *Arabidopsis thaliana* plants were washed with destilled water and a part of approximately 1 cm x 1 cm was cut out with a sharp scalpel. Plant material together with some corns of quartz sand and 50 µL CTAB-buffer were transferred to an Eppendorf tube and grinded with a glass rod to a homogeneous suspension. To collect residual plant material remained on the glass rod, 50 µL CTAB-buffer were pipetted on it and collected in the same tube. Afterwards samples were vortexed, shortly centrifuged and incubated on a thermo shaker for 30 minutes, 1000 rpm at 60°C. The same volume chloroform:isopropanol (24:1) than the sample was applied to the homogenous suspension and mixed for 30 seconds by vortexing. After centrifugation for 10 minutes, 10000 rpm at room temperature the supernatant was transferred to a fresh Eppendorf tube and another chloroform:isopropanol (24:1) extraction was performed as described above. The supernatant transferred to a fresh 1,5 mL Eppendorf tube was mixed with 70 µL (pre-cooled at –20°C) 2-propanol/ isopropanol and chilled on ice for 10 minutes before starting another centrifugation step (10 minutes, 10000 rpm at 4°C). The supernatant was discarded and the DNA pellet was washed with 200 µL 70% EtOH (pre-cooled at –20°C), while the alcohol was added carefully without disturbing the pellet. After centrifugation for 10 minutes, 10000 rpm at 4°C the alcohol was discarded and the Eppendorf tubes were kept open at 37°C to allow evaporation of residual ethanol. The dry pellet was dissolved in 25 µL TE-solution and resuspended by flipping with fingers.

For analysis via gel electrophoresis 2 µL isolated DNA were mixed with 1 µL TAE loading dye and 4 µL ddH_2O^a, while alongside the samples, 3 µL Lamda/ pst DNA were loaded on the agarose gel. Residual DNA was stored at -20°C till further use.

6.6. DNA isolation/ DNeasy Miniprotocol QIAGEN

For DNA Isolation via DNeasy Plant Mini Protocol 100 mg plant material were frozen and ground under $N_{2\ liquid}$ to a fine powder by using a clean mortar and pestle. Immediately 400 µL buffer AP1 and 4 µL of RNase A stock solution (100 mg/ mL) were added to the ground tissue, vortexed vigorously to a homogenous suspension and transferred to an Eppendorf tube. To lyse the cells samples were incubated for 10 minutes at 65° and mixed by inverting the tubes during incubation. To further precipitate detergents, proteins and polysaccharides in the suspension 130 µL buffer AP2 were added to the lysate, mixed and incubated for 5 minutes on ice. To obtain optimal results the lysate was centrifuged for another 5 minutes at 13000 rpm before applying the supernatant to a QIAshredder Mini Spin Column, placed in a 2 mL collection tube. After centrifugation for 2 minutes at 13000 rpm the flow-through fraction was applied to a fresh 1,5 mL Eppendorf tube without disturbing the pellet. 1,5 volumes of buffer AP3/ EtOH were added to the cleared lysate and mixed, while 650 µL of this mixture, including any precipitate which may have been formed were applied to the DNeasy Mini Spin Column, sitting in a 2 mL collection tube. After centrifugation for 1 minute at 8000 rpm the flow-through was discarded and the step repeated with the residual sample. The column was replaced in a fresh 2 mL collection tube and 500 µL buffer AW were added. The flow-through was discarded after centrifugation for 1minute at 8000 rpm and the collection tube was reused in the next step. Another washing step with 500 µL buffer AW was performed followed by centrifugation for 2 minutes at 14000 rpm. The dry column was transferred to a fresh Eppendorf tube and 100 µL of preheated (65°C) buffer AE were pipetted directly onto the DNeasy membrane to elute the DNA. After incubation for 5 minutes at room temperature the samples were centrifuged for 1 minute at 8000 rpm. To increase the final DNA yield the previous elution step was repeated.

For further gel electrophoresis or photometric measurement, it was necessary to concentrate the isolated DNA using a Classic SpeedVac. This apparatus is a combination of centrifugal force, heat and vacuum to dry multiple samples in a single run. After this procedure 20 µL ddH2Oa were added to the sample and resuspended thoroughly.

2 µL concentrated DNA were mixed with 1 µL TAE loading dye and 4 µL ddH_2O^a for analysis via gel electrophoresis, while alongside the DNA samples, 3 µL Lamda/ pst DNA were loaded on the agarose gel. Residual DNA was stored at -20°C till further use.

6.7. RNA isolation/ RNeasy Miniprotocol QIAGEN

For isolation via RNeasy Miniprotocol (QIAGEN) 100 mg plant material and one to two beads (Precellys-Stahl-Kit 2,8 mm) were transferred to an Eppendorf tube, frozen in $N_{2\ liquid}$ and ground using a Tissue Lyser. Immediately 600 µL RLT buffer supplemented with β-ME (10 µL β-ME / 1mL RLT buffer) were applied to the pulverized plant material and vortexed to a homogenous suspension, to protect samples from degradation. The lysate was transferred to the QIAshredder Spin Column and after centrifugation for 2 minutes at 13000 rpm the inlet was taken out, while the flow through fraction was transferred to a fresh Eppendorf tube without disturbing the cell debris. 0,5x volume absolute ethanol were applied immediately, mixed by pipetting and transferred to the RNeasy Mini Column. After centrifugation for 1 minute at 13000 rpm the flow through fraction was discarded and the inlet replaced into the 2 mL collection tube. The membrane in the column was once washed with 700 µL RW1 buffer and after centrifugation for 1 minute at 13000 rpm twice with 500 µL RPE buffer. After centrifugation for 2 minutes at maximal rotation speed, the column was transferred to a fresh Eppendorf tube and total RNA was eluted with 30 µL RNase free water. To provide higher RNA concentration for further analysis, the elution step was repeated after centrifugation for 1 minute at 13000 rpm. Both flow through fractions were pooled and mixed before aliquoting 2x 5 µL of the sample for further analysis via agarose gel electrophoresis and photometric measurement.

For analysis of total RNA via gel electrophoresis an 0,8% agarose gel (0,24 g agarose, 30 mL buffer (50x TAE with ddH_2O^a supplemented with DEPC, 1 µL EtBr)) was prepared, using the same buffer as a running buffer. Before loading the samples, 2 µL of a special RNA loading dye (460 µL 87% glycerol were mixed with 40 µL RNAse free water; a little amount of bromphenol blue was applied) were added. As a length marker 3 µl Lambda/pst were loaded alongside the samples and the gel was run with ~65 V till the samples have migrated approximately 4 cm on the gel.

Residual samples of isolated, total RNA (50 µL) were frozen in $N_{2\ liquid}$ and stored at –80°C till further analysis.

6.8. Polymerase chain reaction (PCR)

PCR is a technique used to amplify small amounts of template DNA exponentially. The process requires several basic components, including: DNA template, comprising the desired DNA fragment; primers, which determine the part to be amplified by providing the starting points for the synthesis of a new DNA strand; Go *Taq* Polymerase, a thermostable enzyme which polymerizes the desired DNA fragment, and for providing a suitable environment for the polymerase a buffer has to be applied.

Almost all primers used for this project, were designed at www.sgd.com , checked manually and ordered at http://www.vbc-biotech.at/cms/index.php.

The PCR process works in three steps and is accomplished in a thermocycler, which raises and eases the temperature for each step of the reaction. Initially double stranded DNA is heated to 94 - 95°C for three to five minutes to separate the Watson and Crick strand by breaking hydrogen bonds. Afterwards, 25-30 cycles comprising denaturation at 94 - 95°C for 30 seconds, followed by primer annealing at primer specific temperature for 30 seconds, and strand elongation at polymerase specific temperature are run. The elongation time depends on the length of the fragment to be amplified and on the amplification speed of the polymerase used (1 kb/ min in case of Go *Taq* Polymerase). The temperature used for primer annealing depends on the length of the oligonucleotides, and the ratio of AT/ GC nucleotides. During primer annealing, they hybridize to the single stranded DNA template, after which strand elongation begins, where the Go *Taq* Polymerase starts to polymerize the DNA template. This step is run at 72°C which is the optimum temperature for Go *Taq* Polymerase which elongates the reverse and forward primer, therefore exponentially replicating the DNA template and producing enough copies needed for further analysis.

For every PCR reaction performed during this project, wild type DNA or RNA was used as a positive control, while ddH_2O^a or ddH_2O^a supplemented with DEPC instead of nucleotides was used as a negative control. Finally all PCR products were separated on an agarose gel and visualized by EtBr staining.

6.8.1. Genotyping of T2 plants, backcrosses and their progenies

For genotyping of T2 plants, backcrosses and their progenies (Fig. 62) genomic DNA was isolated via CTAB-method or DNeasy Plant Mini Protocol, respectively and PCR reactions

were performed under standard conditions, using following primer pairs (Fig. 10, orange arrows) and PCR programs: T-DNA LB 5'-CCCATTTGGACGTGAATGTA GACA-3' (primer forward) and 814C11 5'-TTGTTCCTGTGGTTATTGGTA-3' (primer reverse), yielding one band approximately 800 bp in size. PCR was performed with an initial denaturation of 95°C for five minutes, followed by 35 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for one minute, finishing with an extension at 72°C for five minutes. Those primers were almost used for genotyping of T2, BX1 plants and their progenies (Fig. 62).

For checking BX2, BX3 and their progenies (Fig. 62), following primer pairs (Fig. 10, green arrows) were designed: GSP 5'-TTGATTACTTCTCCCAGTTGGCT-3' (primer forward) and T-DNA LB/2 5'-GCTGCG GACATCTACATTTTTG-3' (primer reverse), yielding one band 382 bp in size. PCR was performed with an initial denaturation of 95°C for three minutes, followed by 33 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 30 seconds, finishing with an extension at 72°C for five minutes. To double check the results amplified by PCR, another round of genotyping with following primers (Fig. 10, black arrows) was performed: RB 5'-GTAATATCCGGAAAC CTCCTCG-3' (primer forward) and GSP/ 2 5'-CCTTTGGTAACTCACTGGTGTGAT-3' (primer reverse), yielding one band 552 bp in size. PCR was performed with an initial denaturation of 95°C for three minutes, followed by 35 cycles of 95°C for 30 seconds, 50,5°C for 30 seconds, and 72°C for 35 seconds, finishing with an extension at 72°C for five minutes.

Additionally gene specific primers (Fig. 10, lilac arrows) were used for genotyping on genomic DNA level: At3g12760 for 5'-AAGTGAAGAAGCAATCGT-3' (primer forward) and At3g12760 rev 5'- TGAGATAAGCAAGTCTAT-3' (primer reverse), yielding one band approximately 2132 bp in size. PCR was performed with an initial denaturation of 95°C for five minutes, followed by 30 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 90 seconds, finishing with an extension at 72°C for five minutes.

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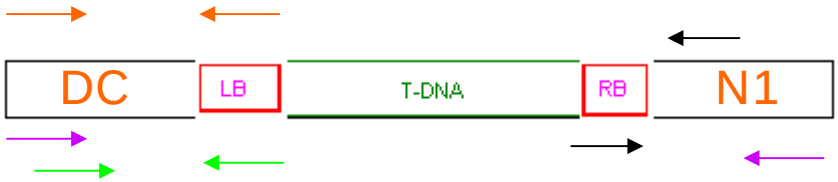


Fig. 10 Primers used for Genotyping on gDNA level
Orange arrows: T-DNA LB and 814C11 primers; **green arrows**: GSP and T-DNA LB/2 primers; **black arrows**: RB and GSP/2 primers and **lilac arrows**: At3g12760 for and rev primers

6.8.2. Preparation of isolated, total RNA for RT-PCR via DNase I digestion

Total RNA was extracted from leaves or seedlings using RNeasy Plant Mini Kit (QIAGEN) and digested with DNase I to ensure good conditions for further RT-PCR. 1 µL 10x buffer for DNase I, 1 µL DNAse I, 1 µL RNA (1 µg/ µL) and 7 µL RNase-free water were mixed and incubated for 30 minutes at 37°C. To stop the enzymatic reaction 1 µl EDTA was applied and after another incubation of 10 minutes at 65°C the RNA samples were free of any residual DNA and ready for further cDNA synthesis via RT-PCR.

6.8.3. Expression analysis of backcrosses and their progenies via RT-PCR

For cDNA synthesis via RT-PCR two Mastermixes were prepared as follows:

Mastermix 1		Mastermix 2	
5,5 µL	H_2O (RNase free)	5,5 µL	H_2O (RNase free)
1 µL	dNTPs	4 µL	5x RT Buffer
1 µL	Random primer (Hexamers)	1 µL	DTT
		1 µL	RNasin
		0,25 µL	AMV-RT

1µL DNase I digested RNA was added to 7,5 µL of Mastermix 1, incubated for 5 minutes at 65°C and chilled for three minutes on ice before 11,75 µL of Mastermix 2 were applied. The samples were briefly centrifuged and left for five minutes at room temperature followed by incubation for two hours at 42°C, which is the optimum temperature for AMV-RT. To stop the enzymatic reaction the Eppendorf tubes were incubated at 72°C for 15 minutes.

As a control, cDNA was always checked via PCR using 18s primers: 18S-5’ 5’-CGTAG TAATTCTAGAGCTAATACGTGC-3’ (primer forward) and 18S-3’ 5’-CTCATTCCAATT ACCAGACTCATAGAGC-3’ (primer reverse), yielding one band approximately 300-400 bp in size. PCR was performed with an initial denaturation of 94°C for two minutes, followed by 27 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, finishing with an extension at 72°C for 5 minutes.

To check the presence of an *AtDCN*1 (At3g12760) transcript via PCR on cDNA level following primer pairs were used: At3g12760 for 5'-AAGTGAAGAA GCAATCGT-3' (primer forward) and At3g12760 rev 5'- TGAGATAAGCAAGTCTAT-3' (primer reverse), yielding one band 1022 bp in size, if a proper transcript is present. If the T-DNA insertion in this mutant line is located within *AtDCN*1, we would expect no band after amplification with those primers. PCR was performed with an initial denaturation of 95°C for five minutes, followed by 35 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 90 seconds, finishing with an extension at 72°C for five minutes.

GABI-Kat mentioned a second insertion in this mutant line to be located within At2g28540 or At3g12770 (*AAR*3), respectively, which was proved via PCR on transcript level using following gene specific primer pairs: At2g28540 for 5'-CGTTGTCCTGCATGTCGTACCCG TT-3' (primer forward) and At2g28540 rev 5'-CCAGTGACCATTAGGCAAGGCCATA-3' (primer reverse), yielding one band 2655 bp in size; and At3g12770for 5'-GCTCAGCTGAA GCAAATCCACGCACGTT-3' (primer forward) and At3g12770rev 5'-CGCAATCGCGA TCCTCTCGCTGT-3' (primer reverse), yielding one band 1797 bp in size. To proof the presence of both transcripts, PCR was performed with an initial denaturation of 94°C for two minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for two minutes, finishing with an extension at 72°C for two minutes.

6.8.4. PCR approaches to check for multiple insertions within *AtDCN*1

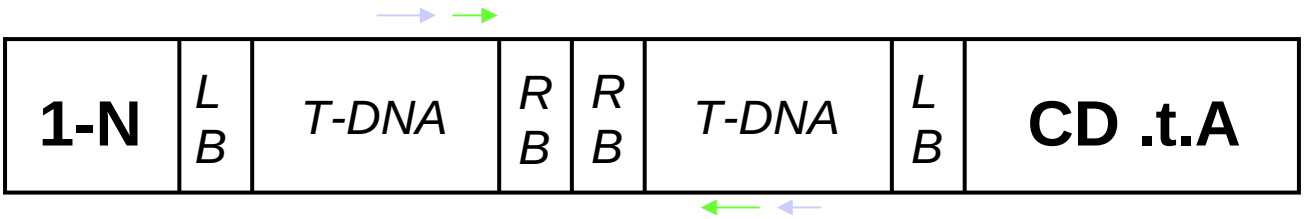


Fig. 11 Primers used for checking the presence of multiple insertions within At3g12760

To determine the possible presence of two T-DNA insertions within At3g12760, genomic DNA was isolated from leaves using DNeasy Plant Mini Protocol and PCR reactions were performed under standard conditions, using following primer pairs (Fig. 11, green arrows) and PCR programs: RB fwd 400 5'-TTCGCAAGACCCTTCCTCTATA-3' (primer forward) and RB rev 400 5'-TG CTCTCGTAGCACCTTTTT-3' (primer reverse), yielding one band with

371 bp in size. PCR was performed with an initial denaturation of 95°C for five minutes, followed by 35 cycles of 95°C for 30 seconds, 49°C for 30 seconds, and 72°C for 90 seconds, finishing with an extension at 72°C for five minutes. To assure those results another primer pair (Fig. 11, blue arrows) with similar purpose was designed and used for amplification via PCR as follows: RB fwd 900 5'-GAC AGTGGTCCCAAAGATGGA-3' (primer forward) and RB rev 900 5'-CGGTCACTT AACTAGGGGTTAA-3' (primer reverse), yielding one band 902 bp in size. PCR was performed with an initial denaturation of 95°C for five minutes, followed by 35 cycles of 95°C for 30 seconds, 45°C for 30 seconds, and 72°C for 90 seconds, finishing with an extension at 72°C for five minutes.

6.8.5. Check the expression of truncated versions of an *AtDCN*1 transcript

Fig. 12 Primers used to check the expression of truncated versions of an At3g12760 transcript

To check the expression of truncated versions of an *AtDCN*1 transcript RNA was isolated from leaves via RNeasy Miniprotocol (QIAGEN) and cDNA was synthesized as described in Materials and Methods. PCR reactions were performed under standard conditions, using following primer pairs and PCR programs: Ende fwd 5'-AGTGCTGTCGAATTACGAT GCA-3' (primer forward) and Ende rev 5'-CACTTCTCAACGACATTTTTGTCA-3' (primer reverse) (Fig. 12, pink arrows), yielding one band 93 bp in size. PCR was performed with an initial denaturation of 95°C for three minutes, followed by 30 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds, finishing with an extension at 72°C for five minutes. Anfang fwd 5'-ATAAGTTGAGCAGAAGCA ACCGT-3' (primer forward) and Anfang rev 5'-CCTCCGCAAGAATCATATCAGA-3' (primer reverse) (Fig. 12, yellow arrows), yielding one band 231 bp in size. PCR was performed with an initial denaturation of 95°C for three minutes, followed by 33 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 30 seconds, finishing with an extension at 72°C for five minutes.

6.8.6. Amplification of Southern blot probes

For amplification of two Southern blot probes designed to bind to the left boarder region of the T-DNA insertion following primer pairs were designed: p106_f 5'-CGTGATTGATGC TGTTGAGTTACCAA-3' (primer forward) and p106_r 5'-GCCAGAGACCGAGGGTTAGA TCAT-3' (primer reverse), yielding one band 344 bp in size (first LB probe). LB fwd 5'-GTGTCTACATTCACGTCCAAA-3' (primer forward) and LB rev 5'-ACCCGGGGATCA GATCTTATA-3' (primer reverse), yielding a PCR product of 476 bp in size (second LB probe). PCR was performed with an initial denaturation of 94°C for five minutes, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds, finishing with an extension at 72°C for five minutes.

For amplification of the RB Southern blot probe, following primers were used: pp1 fwd 5'-CATGCAAGCTTGGCGTAAT-3' (primer forward) and pp1 rev 5'-TATGGAAAAACG CCAGCAAC-3' (primer reverse), yielding a PCR product of 433 bp in size. PCR was performed with an initial denaturation of 94°C for five minutes, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, finishing with an extension at 72°C for five minutes.

PCR mixtures were loaded on an 0,8% agarose gel and visualized by ethidium bromide staining. The proper bands were cut out of the gel, purified using Wizard SV Gel and PCR Purification System and used for further radioactive labelling and hybridization of the DNA blots.

6.8.7. Amplification of Northern blot probe

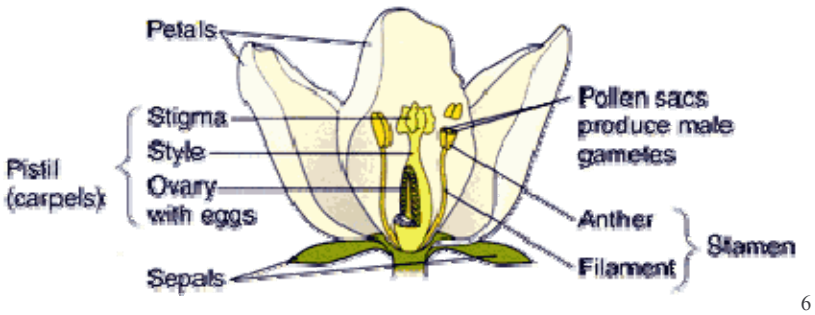
For hybridization of RNA blots during this project a probe amplified from wild type col-O cDNA, using following primers was used: At3g12760 for 5'-AAGTGAAGAAGCAATCGT-3' (primer forward) and At3g12760 rev 5'- TGAGATAAGCAAGTCTAT-3' (primer reverse), yielding one band approximately 1022 bp in size. PCR was performed with an initial denaturation of 95°C for five minutes, followed by 35 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 90 seconds, finishing with an extension at 72°C for five minutes. The whole PCR mixture was loaded on an 0,8% agarose gel and visualized by ethidium bromide staining. The proper band was cut out of the gel, purified using Wizard SV Gel and

PCR Purification System and used for further radioactive labelling and hybridization of the Northern blots.

6.9. Wizard SV Gel and PCR Purification System

The Wizard® SV Gel and PCR Clean-Up System was designed to extract and purify DNA fragments directly from PCR reactions or from agarose gels[5]. After amplification via PCR followed by gel electrophoresis, a proper band was cut out of a preparative gel and 10 µL membrane binding solution per 10 mg agarose gel slice were added and incubated at 65°C until agarose slices were melted. The solution was then transferred to a DNA binding column stacked into a collection tube. Binding of DNA happened while centrifugation at maximum speed for 1 minute. The flow through was discarded and the samples were washed twice with 700 µL and 500 µL membrane wash solution under centrifugation steps as described above. The DNA binding column was transferred to a fresh 1,5 mL Eppendorf tube and after application of 50 µL nuclease-free water and incubation for one minute at room temperature, the DNA was eluted by centrifugation at maximum speed for one minute. The quantity and purity of the purified DNA was determined by gel electrophoresis and residual samples were stored at –20°C until further use.

6.10. Backcrossing of *AtDCN*1 T-DNA insertion lines to a wild type background

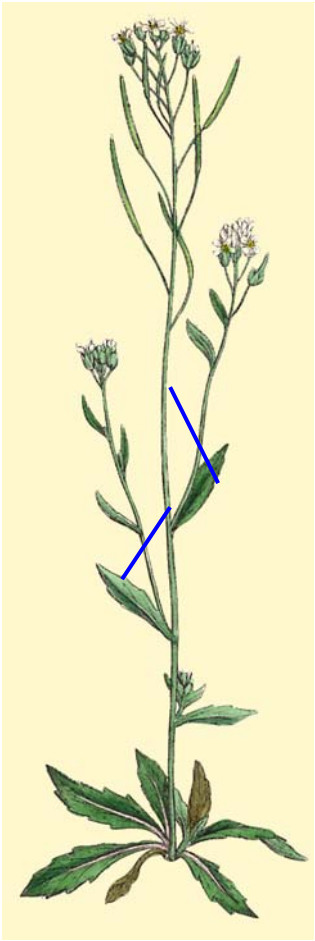


6

Fig. 13 Parts of an *Arabidopsis thaliana* flower

Backcrossing in order to cross out multiple insertions in a plants genome was performed as follows:

[5] http://www.promega.com/applications/pcr/featuresandbenefits/Wizard_SV_Gel_PCR_Clean-Up_System.htm
[6] http://scienceblogs.com/pharyngula/2006/11/mads_boxes_flower_development.php



In the individual plant (wild type or mutant plant/ female parent) chosen for pollination all siliques and lower side shoots (Fig. 14, see blue marks) were abscised to concentrate the plant's energy on generating healthy backcrossed seeds. Female donor plant was prepared for pollination by removing sepals, petals and anthers with a forceps from approximately five flowers per plant, using a stereo microscope. The flowers for emasculation should to be at the stage just before they open (the white petals are becoming to be visible). If the flowers are too immature the stigma will not mature, whereas when flowers are too open the flower is most likely already fertilized. Flowers were opened by carefully squeezing with the forceps. The anthers should be below the stigma; at this stage they are not mature and the pollen is not shed. The backcrossing process was performed very carefully, without damaging the stigma, style or vascular tissue; otherwise the cross would not be successful. Healthy plants usually have three flowers on the main shoot suitable for crossing. Two to five plants (approximately five flowers per plant) were backcrossed to ensure that enough material will be obtained. Emasculated flowers were marked with an aluminium foil or the similar material.

Fig. 14 *Arabidopsis thaliana* plant

A plant line (wild type or mutant plant/male parent) chosen as a male donor should have open flowers and under the stereo microscope the anthers shedding pollen must be visible. A flower of this plant was abscised with a forceps at the same time squeezing the flower near the base so that it "opens up". The backcross was performed by tapping this open flower on the emasculated stigma of the female part, so that its pollen is sticking on the stigma. As a negative control one emasculated stigma per plant was kept unpollinated and should not further develop into a mature silique.

If the pollination has been successful, the style will have elongated and a silique will start to develop. Two to three weeks later the siliques became yellow and were collected separately in different bags, in case that not all backcrosses of one plant worked out. The seeds were dried at room temperature for approximately two weeks before planting.

[7] http://ec.europa.eu/research/quality-of-life/image/arabidopsis.jpg

6.11. Southern blot analysis

The first step of Southern blot analysis was the digestion of genomic DNA into small fragments by restriction enzymes. Therefore a Mastermix of restriction enzymes, ddH_2O^a and proper buffer was mixed with genomic DNA (8-10 µg) to be analysed and was incubated over night at certain temperature specific for the restriction enzyme which was applied.

Next day the digested DNA was run on an 0,8% agarose/ 1x TAE gel (100 ml 1x TAE, 0,8 g agarose, 1,4 µL EtBr) for two to three hours with approximately 65 V, while a Transiluminator was used to take a picture of the gel including a ruler for further estimation of the sizes of appearing signals. The gel was cut to appropriate size keeping the gel slots and was incubated at room temperature with agitation for 10 minutes in 0,25 M HCl, for 1 minute in ddH_2O^a, three times for 10 minutes in denaturation solution, for 1 minute in ddH_2O^a and twice for 15 minutes in neutralisation solution.

After preparing of the gel by incubation in different solutions, the blot was set up (Fig. 15) using 10x SSC as a transfer buffer as follows: A plastic tray and electrophoresis carriage were cleaned properly with soap and 70% ethanol, three Whatman papers were cut in the size of the gel and in the size of approximately 25 x 13 cm (depends on the size of the plastic tray), respectively. The plastic tray was filled with 10x SSC and the electrophoresis carriage was placed on the tray. The Whatman papers, cut in the size of 25 x 13 cm were placed over the carriage, allowing the ends of the Whatman papers to drunk into the transfer buffer. The gel was placed with the backside up on the Whatman papers, while gaps between the plastic tray and the carriage were sealed with Parafilm to avoid the transfer buffer to evaporate while incubation. Carefully a Nylon membrane was transferred on the blot and at the backside of one edge, the membrane was marked with a pencil. The Whatman papers, cut in the size of the gel and 5 cm of green towels (cut in size of membrane) were placed on the membrane, while a weight of approximately one kilogram placed on a glass plates was used to stabilize the blot during over night at room temperature.

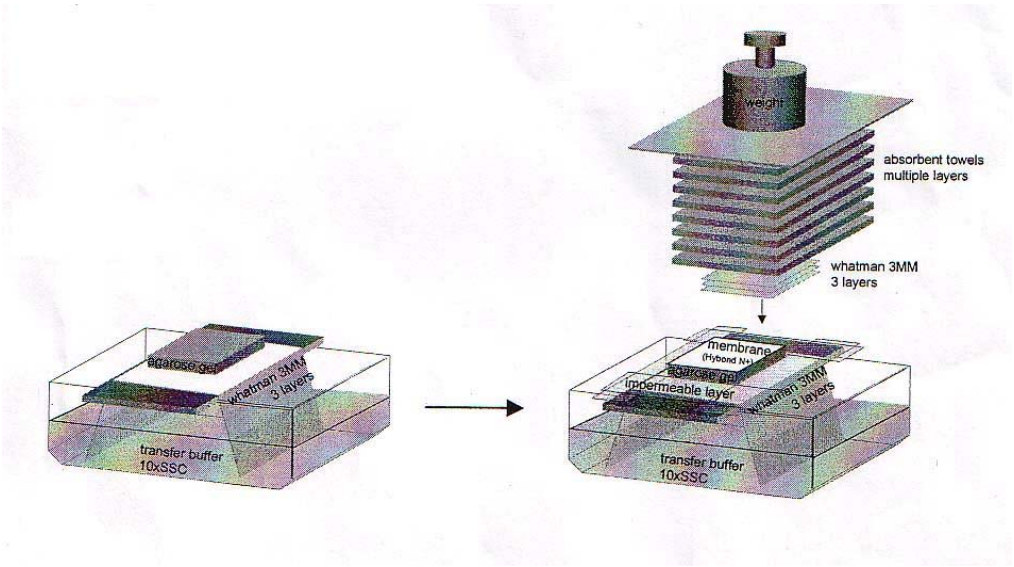


Fig. 15 Set up Southern blot

The next day the blot was dissembled and the gel slots were marked on the membrane with a pencil. The membrane was wrapped in saran wrap and its non-labelled side (DNA side) was exposed to 100% UV for 2 minutes.

For pre-hybridization the membrane was unwrapped and incubated in 100 mL 2x SSC with agitation for at least 2 minutes at room temperature before transferring the membrane into a cleaned, dried hybridization tube (labelled non-DNA side should show outside). To reduce the background signal of the Southern blot, the membrane was pre-hybridized with denatured salmon sperm DNA (150 µL Salmon sperm DNA incubated for 10 minutes at 96°C) and 15 mL church buffer for at least one hour at 65°C with middle rotation speed of the hybridization oven.

During incubation of pre-hybridization the Southern probe was thawed on ice, diluted (e.g. 2 µL probe + 19 µL ddH_2O^a) and incubated for 10 minutes at 96°C. For radioactive labelling of the probe following components were added: 1 µL 500 µM dATP, 1 µL 500 µM dGTP, 1 µL 500 µM dTTP, 20 µL 2,5x Random Primer Solution, 1 µL Klenow fragment and 5 µL [α-^{32}P] dCTP. This mixture was incubated for 10 minutes at 37°C, which is the optimum temperature for the Klenow fragment. Before applying 5 µL Stop buffer (Rad Prime Labelingsystem; 0,5 M EDTA pH 8) the samples were chilled on ice.

After pre-hybridization the membrane was hybridized with the labelled, denatured probe (10 minutes at 96°C), 100 µL denatured salmon sperm DNA (10 minutes at 96°C) and 10 mL church buffer over night at 65°C with fourth rotation-speed of the hybridization oven.

Next day the membrane was washed with following buffers, each time using 10 mL: Twice with 2x SSC/0,1%SDS for seven minutes, one time with SSC/0,1%SDS for 30 minutes

and twice with 0,1x SSC/0,1%SDS for 10 minutes. Afterwards the membrane was taken out of the hybridization tubes and was dabbed off between green towels.

For further analysis a film cassette was covered with an autoclave sack, the membrane was packed into it and fixed with tape, while residual radioactive radiation was detected using a geiger counter. A film was applied in the darkroom, the film cassette was closed properly and the cassette was incubated at -80°C for the suggested time according to detected cps. After incubation at –80°C the film cassette was thawed at room temperature, opened in the darkroom and the film was developed by using a Curix (film developer).

200-100cps	1h	@ -80°C
50cps	2hrs	
20cps	3-5hrs	
10cps	10hrs	
5cps	o/n	

The membrane, hybridized with the radioactive labelled probe can be reused e.g. for hybridization with another radioactive labelled probe as a control, after stripping the membrane as follows: The membrane was placed in a cleaned glass tray with the non-labelled DNA side up, while 0,1% SDS was boiled up and poured over the membrane. The solution was incubated till it was chilled, the membrane was taken out, dabbed off between green towels and was stored at 4°C wrapped in saran foil till another Southern blot was performed by starting with the pre-hybridization step.

6.12. Northern blot analysis

For analysis via Northern blotting, RNA was isolated from leaves or seedlings, respectively using RNeasy Miniprotocol (QIAGEN), while 10-15 µg/ 6 µL RNA were used for further gel electrophoresis. For preparation of the gel, 1,5 g agarose were weighed in a proper Erlenmeyer vial and 75 mL ddH_2O^a supplemented with DEPC were applied with a sterile pipette. This mixture was then heated in a microwave to melt the agarose and cooled down to 55-60°C on a magnetic stirrer with 200-500 rpm for further preparation. Using a glass pipette, 10 mL 10x MOPS buffer and 17 mL formaldehyde were applied at the wall-stream of the vial and the still liquid gel was poured in a plastic electrophoresis tray equipped with a comb and was left under a fume hood at room temperature for polymerization. Before loading the gel RNA samples have to be prepared as follows: 10-15 µg/ 6 µL RNA were mixed with the same

volume 2x RNA loading dye solution (for RNA gel electrophoresis, FERMENTAS) and after incubation for 10 minutes at 65°C the samples were chilled on ice for two minutes. After short centrifugation the samples were loaded carefully on the 1,5% agarose gel using 1x MOPS as a running buffer and electrophoresis was performed at 35-40 V for three and a half to four hours. The gel was examined and photographed using a Transiluminator followed by equilibration of the gel in 10x SSC with ddH_2O^a supplemented with DEPC for maximum 30 minutes on shaker.

The blot was set up (Fig. 15) as described for Southern blot analysis, using 20x SSC supplemented with DEPC as a transfer buffer.

After dissembling the blot on the next day, the membrane was packed between two Whatman papers and baked with the DNA-side up on a gel dryer for two and a half hours at 80°C without vacuum. Pre-hybridization, labelling of the probe and hybridization steps were performed as described for Southern blot analysis.

6.13. Western blot analysis

For Western blot analysis proteins were isolated from approximately two weeks old seedlings as follows: 200 mg frozen plant material were ground in 100 µL Lacus buffer and 50 µL sand with a pre-cooled mortar and pistil. The pulverized material was collected and transferred into a pre-chilled Eppendorf tube. After vortexing properly, the samples were centrifuged for 40 minutes, 14000 rpm at 4°C and the supernatant was transferred into a fresh tube. To measure protein concentrations with Bradford 2 µL protein extract, 798 µL ddH_2O^a and 200 µL Biorad reagents were mixed, transferred into a plastic cuvette and measured using program 13 on the photometer (595 nm).

For performing SDS-PAGE, the electrophoresis equipment was set up, the separating gel was filled in and a few amount of ddH_2O^a was added to straight the surface of the gel. After polymerization the water was removed, the stacking gel was prepared and poured onto the separating gel after placing a comp in the equipment to generate slots for loading the protein samples, which were prepared as follows: 6x sample buffer were mixed with 1/ 20 β-ME whereof 1,7 µL were added to 10 µg protein solution and filled up with ddH_2O^a to an final volume of 10 µL. Samples were heated for three minutes at 95°C, while the whole amount of 10 µL was loaded on the SDS-PAGE, using 3 µL PageRuler^{TM} Plus Prestained Protein Ladder as a protein marker. The separating gel was run with 130 V, the stacking gel with 150-170 V

using 1x SDS as a running buffer. After stopping the run the electrophoresis apparatus was dismantled and the gel incubated in 1x TBE for 15 minutes at room temperature.

For preparation of the Western blot a piece of PVDF membrane was cut to proper size (~7x 9 cm), pulled through methanol and incubated for five minutes in 1x TBE buffer. Before assembling the blotting apparatus (Fig. 16), four pieces of three millimetre Whatman paper (~7x 9 cm) were prepared and proper sponges and Whatman papers were pre-wet in 1x TBE buffer. The transfer was incubated for one hour at 4°C, 250mV on a stir plate, using 1x TBE buffer. After incubation the blotting apparatus was dissembled and the membrane was washed in 1x PBS for five minutes. Before signal detection via antibodies, the membrane was blocked in blocking solution (1000 mL 1x PBS, 50 µL TWEEN-20, 30 g BSA) with agitation for two hours at room temperature, afterwards incubating the membrane with the primary antibody (15 mL blocking solution with 15 µL antibody/HA-Ab, diluted 1:5000 in blocking solution) over night at 4°C. After washing the membrane five times with 1x PBST, for ten minutes at room temperature with agitation the membrane was incubated with the 2nd antibody (anti-mouse alkaline phosphatise conjugate, diluted 1:5000 in blocking solution) for one hour at room temperature with agitation. After another washing step the membrane was incubated for five minutes in CDP star detection reagent..

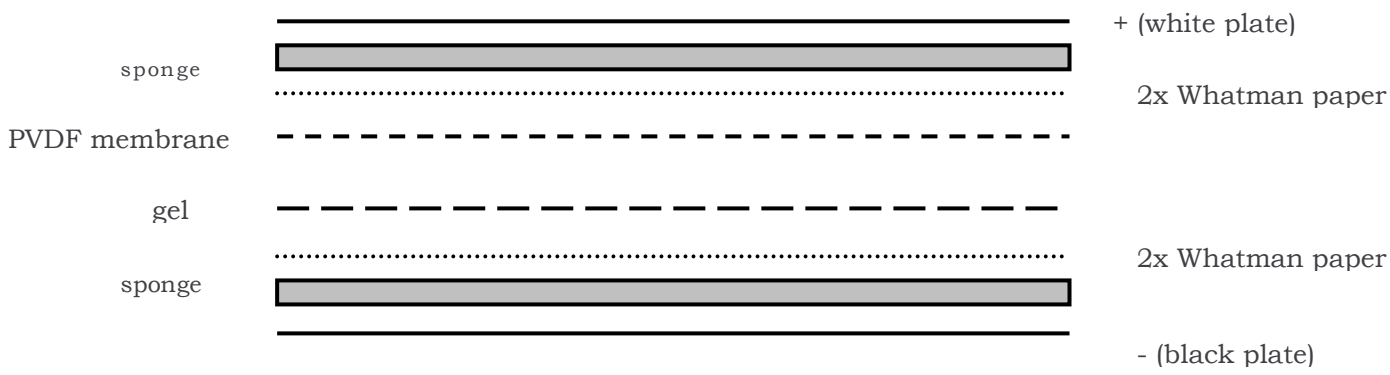


Fig. 16 Set up Western Blot

For further analysis the membrane was packed and fixed in a film cassette covered with an autoclave sack. In the darkroom, a film was applied on the membrane and after incubation for one minute and three minutes, respectively the film was developed using a Curix (film developer).

As a control Coomassie staining was used to visualize protein bands after SDS-PAGE, therefore incubating the membrane in a few millilitre Coomassie dye. Afterwards the

membrane was washed two to three times and left at room temperature to allow drying of the membrane before scanning.

6.14. Phenotypes, germination rates and segregation analysis

For phenotypic analysis of backcrosses, seeds were sterilized with 70% EtOH, germinated in agarose solidified MS medium and further planted in soil, as described in Materials and Methods. Germination rates were determined by calculating the percentage of germinated versus non-germinated seeds, approximately one week after spreading them out in MS medium. Abnormalities in the phenotypes of the backcrosses in contrast to wild type plants were checked from the time of germination till seedlings further developed into plants while mainly observing the size and quantity of the rosette leaves, the over-all height of the plants, as well as the size and form of flowers.

For segregation analysis BX1a and wild type col-O seeds were sterilized and germinated on agarose solidified MS medium containing either 50 mg/ mL Kanamycin or 75 mg/ 10 mL Sulfadiazin, while germination rates were calculated as described above. As a control seeds were germinated on agarose solidified MS medium as well, without addition of any antibiotics.

6.15. DAPI staining of *AtDCN*1 mutant pollen

To collect pollen from interesting backcrosses and an *Arabidopsis thaliana* wild type control, three to four flowers per plant were collected in buffer (50 mM $NaPO_4$, pH 7,1 mM EDTA, 0,1% Triton) and cut longitudinal with a scalpel to free the pollen from the anthers by tapping with a pipette tip on the flowers. To guarantee reproducibility all collected flowers should be in the stage as petals were already visible but still closed, as indicated with a red rectangle in Fig. 17. Before transferring the harvested pollen into an Eppendorf tube for further DAPI staining, residual plant material was removed with a forceps. After centrifugation for five minutes at 2000 rpm the supernatant was discarded, 150 µL (3:1) fixative were added under a fume hood and incubated for at least 15 minutes. During incubation the samples were mixed

Fig. 17 *Arabidopsis thaliana* flowers

two to three times by flipping Eppendorf tubes with fingers and subsequently spinning them down in a centrifuge. As the pollen were fixated the samples were centrifuged for five minutes at 2000 rpm and the supernatant/ fixative was discarded in a special waste. The pellet was washed three times with 300-500 µL 70% EtOH and after removing residual EtOH, at least 20 µL DAPI (~0,5 µg/mL) were added and incubated for approximately 30 minutes at room temperature.

Approximately 20µL of DAPI stained pollen were transferred on a slide, covered with a lid and checked under an UV microscope for any abnormalities in the nuclear structure or organisation of backcrossed pollen in contrast to a wild type control.

6.16. Expression pattern of one *AtDCN*1 homolog and two *AtDCN*1-like genes

For Expression patterning of one *DCN*1 homolog and two *DCN*1-like genes in *Arabidopsis thaliana*, RNA was isolated from root, shoot, stem, flower and leave, respectively using the Tissue Lyser System and RNeasy Miniprotocol (QIAGEN). cDNA was synthesized as described in Materials and Methods and PCR reactions were performed under standard conditions, using following primer pairs and PCR programs: At3g28970 fwd 5'-CCAGTTT CTGCTCGATTCGATA-3' (primer forward) and At3g28970 rev 5'-ATATGTGCGACGAT GTTCCAGT-3' (primers reverse), yielding a PCR product 901 bp in size. At3g12760 fwd 5'-TAAGTTGAGCAGAAGCAAC CGT-3' (primer forward) and At3g12760 rev 5'-AGTTGAT TACTTCTCCCAGTTGGC-3' (primer reverse), yielding one band 775 bp in size. PCRs with both primer pairs were performed with an initial denaturation of 95°C for five minutes, followed by 35 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 90 seconds, finishing with an extension at 72°C for five minutes. At1g15860 fwd 5'-GCTCTTCATCAAA GAAGAAATCAGG-3' (primer forward) and At1g15860 rev 5'-GGCTTGTTTTTCTT GAATCCACTC-3', yielding a PCR product of 674 bp in size. PCR was performed with an initial denaturation of 95°C for five minutes, followed by 33 cycles of 95°C for 30 seconds,

52°C for 30 seconds, and 72°C for 90 seconds, finishing with an extension at 72°C for five minutes. PCR products were separated on an agarose gel and visualized by ethidium bromide staining.

7. RESULTS

7.1. GABI-Kat (Genomanalyse im biologischer System Pflanze)

GABI-Kat is a platform established by University of Bielefeld/ Germany. Using the simple-search function of this homepage, insertions in a favourite *Arabidopsis thaliana* gene can be found easily and seeds from interesting T-DNA insertion lines can be ordered. In case of our gene of interest At3g12760/ *AtDC*N1 (Line-ID 814C11), researches from GABI-Kat infiltrated *Arabidopsis thaliana* wild type col-O plants (referred as T0 plants) with *Agrobacterium tumefaciens* containing vector pAC106 (Fig. 18).

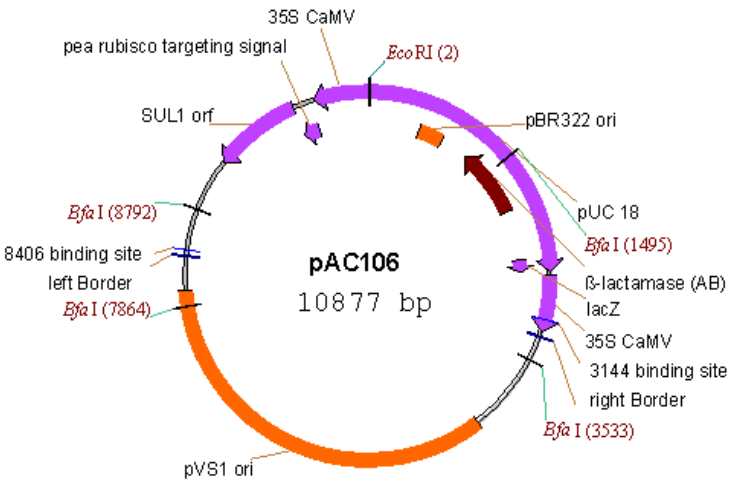


Fig. 18 Map of pAC106 containing the T-DNA
www.gabi-kat.de

The selection for positive transformants occurred via spreading out seeds on agarose solidified MS medium containing Sulfadiazine (SUL), which is the resistance of the vector used (for more information see http://www.gabi-kat.de/faq/sul-selection-scheme.html). Transformed T0 plants produced T1 seeds which were planted and grown to maturity, while genomic DNA isolated from leaves of resistant T1 plants were used for FST (Flanking

Sequence Tags) generation (Strizhov, Li et al. 2003). The T1 generation is usually hemizygous, containing an (intact) T-DNA insertion in their genome and is therefore resistant to SUL. T2 seeds, produced by T1 plants are sent out by GABI-Kat and can be used for functional analysis of the disrupted gene. On their platform GABI-Kat provides some more information about the different T-DNA insertion lines, e.g. information about the vectors used for transformation, primer sequences for genotyping via PCR, as well as FSTs of the mutant lines, which were primed from the LB of the T-DNA present in the vector to your gene of interest.

In case of 814C11 GABI-Kat expected the T-DNA insertion to be located in the 10th exon of At3g12760 as shown in Fig. 19. An alignment of the FST against *AtDCN*1 revealed that the gene is reverse (3'-5') located within the Arabidopsis genome, while the insertion points in a 5'-3' direction (Fig. 20). Furthermore, the 5'- end of At3g12760 overlaps with the 5'- end of the adjacent gene At3g12770 which is shown in Fig.21.

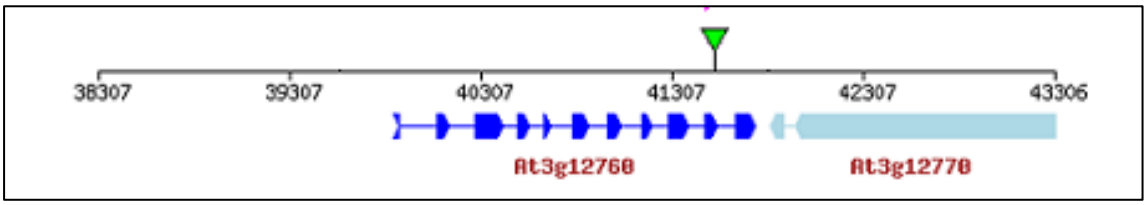


Fig. 19 T-DNA insertion within the 10th exon of At3g12760
http://www.gabi-kat.de/db/picture.php?genecode=At3g12760

Fig. 20 *AtDCN*1 points in a 3'- 5' direction

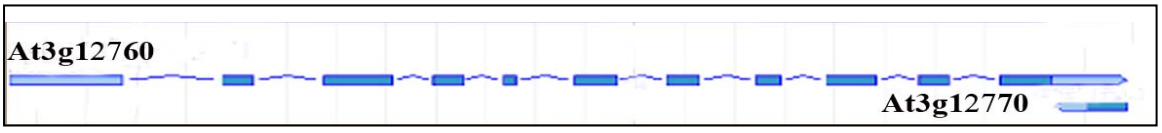


Fig. 21 Protein coding gene model
http://arabidopsis.org/cgi-bin/gbrowse/arabidopsis/?name=AT3G12760

7.2. Initial research on *AtDCN*1 T-DNA insertion line (814C11/ T2 generation)

To determine the primary number of T-DNA insertions within the genome of T2 plants of the 814C11 insertion line, Southern blot analysis was performed as described in Materials and Methods. Genomic DNA from different T2 seedlings was isolated using DNeasy Miniprotocol, was once digested with *EcoRI*, once with P*vuII/HindIII* and blotted on a Nylon membrane. As a probe for radioactive labelling and hybridization a sequence designed to bind to the LB of the T-DNA insertion (first LB probe) was used.

Cut with *EcoRI* Southern blot analysis revealed two (1,7 kb, 2,9 kb for plant 23; 1,9 kb, 2,7 kb for plant 24) up to four (1,7 kb, 1,9 kb, 2,7 kb, 2,9 kb for plants 25 and 27) insertions within the genome of 814C11 mutant plants, while digested with *PvuII/ HindIII* two (2,3 kb, 3,4 kb for plant 24) up to three (2,3 kb, 2,9 kb, 3,4 kb for plants 25 and 27) signals were detected (Fig. 22). Plant 23 showed two insertions when cut with *EcoRI*, but no signal could be detected performing *pVUII/ HindIII* double digestion. Comparing both restriction digests on the blot it can be seen that plant 814C11/24 and 814C11/25 showed the fewest signals, hence containing the fewest insertions within the plant's genome.

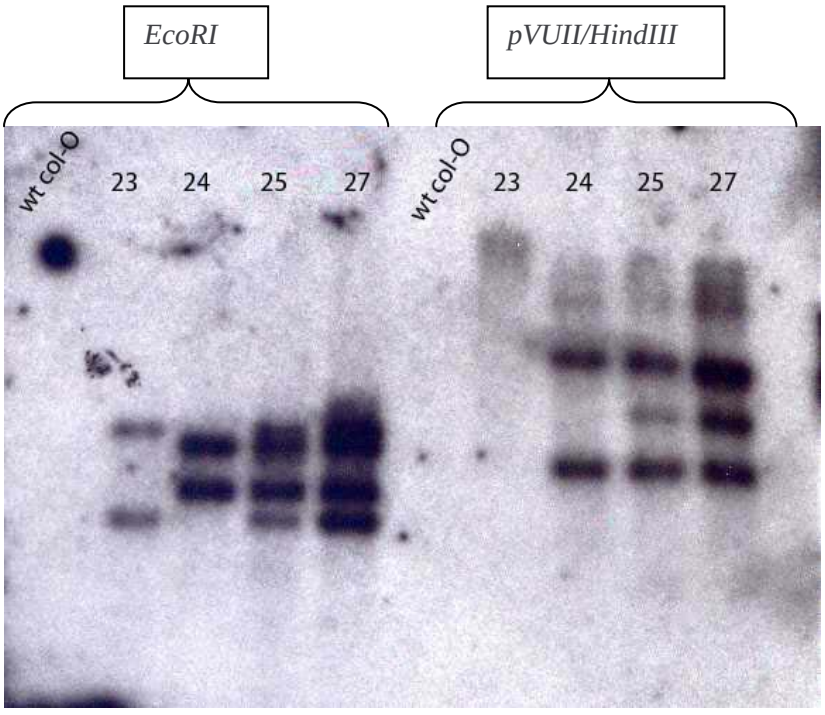
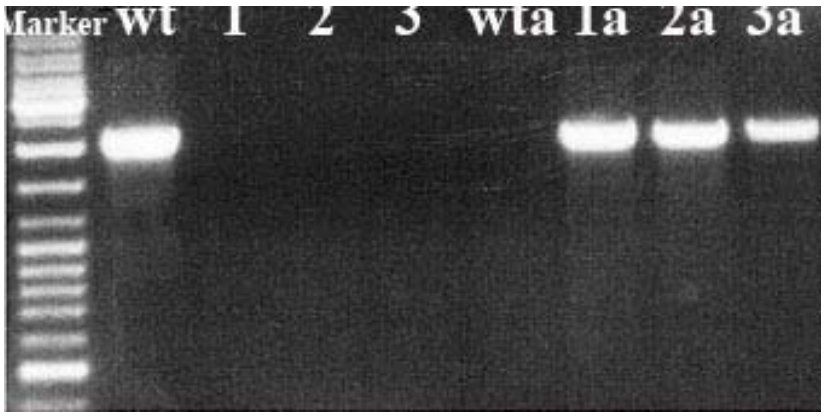


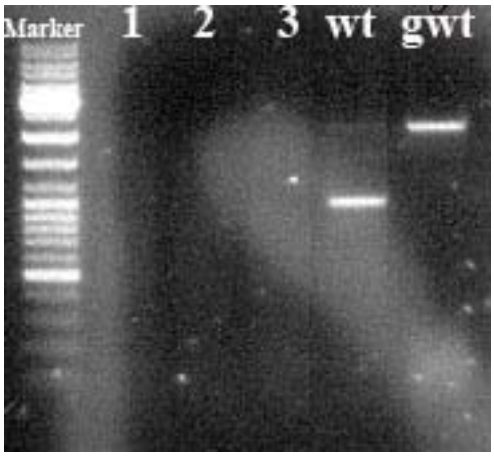
Fig. 22 Southern Blotting
EcoRI and *PvuII/HindIII* digested gDNA (~8-10µg) was run on an 0,8% agarose gel and blotted on a Nylon membrane. Hybridization was performed at 65°C o/n with [α-^{32} P]dCTP- labelled fragments according to the LB region of the T-DNA insertion. Numbers 23,24,25 and 27 represent different plants from the T2 generation of 814C11 mutant line.

For further genotyping of the T2 generation of 814C11 different combinations of primers were used on genomic DNA level, as well as on RNA level for expression analysis. All primary tested plants revealed the presence of a T-DNA insertion within At3g12760, checked by PCR on gDNA level, primed against the LB of the insertion and the gene, as well as with primers specific for At3g12760 (Fig. 23). To approve those results, RNA was isolated and translated to cDNA as described in Materials and Methods and PCR on cDNA level was performed using At3g12760 gene specific primers as well. Results obtained from RT-PCR were very supportive as no transcript could be detected in all checked plants (Fig. 24).



1: T2 plant 24
2: T2 plant 25
3: T2 plant 27

Fig. 23 Genotyping on gDNA level/ T2 plants
O'GeneRuler™ Mix SM1173 (Fermentas) was used as a length marker. gDNA was isolated from seedlings using DNeasy Miniprotocol (QIAGEN). Samples wt, 1, 2 and 3 were amplified with At3g12760 gene specific primers, while the bands appearing in lanes wta, 1a, 2a and 3a are products of PCR, using primers 814C11 and T-DNA LB. PCR products were separated on an 0,8% agarose gel and visualized by ethidium bromide staining.



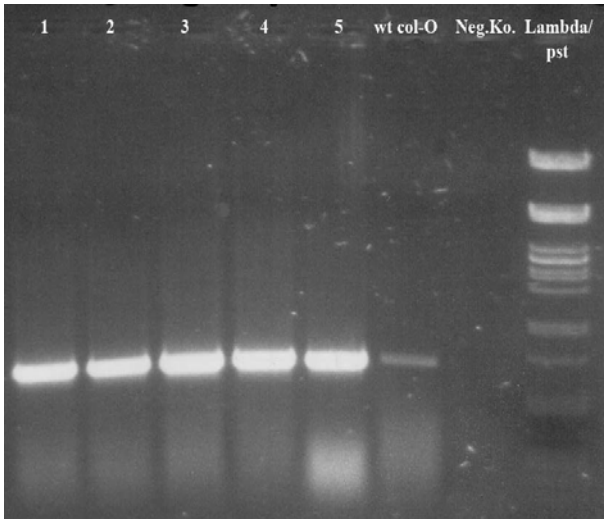
1: T2 plant 24
2: T2 plant 25
3: T2 plant 27

Fig. 24 Expression analysis on cDNA level/ T2 plants
O'GeneRuler™ Mix SM1173 (Fermentas) was used as a length marker. Total RNA was isolated from seedlings using RNeasy Miniprotocol (QIAGEN). All samples were amplified with At3g12760 gene specific primers. Lane wt represents complementary DNA synthesized via AMV-RT, while lane gwt represents genomic DNA. PCR products were separated on an 0,8% agarose gel and visualized by ethidium bromide staining.

7.3. Genotyping of all BX and their progenies

After backcrossing T2 plants of the 814C11 T-DNA insertion line with a wild type col-O background single backcrossed siliques were collected, planted as described in Materials and Methods and grown to maturity for further analysis of their progenies. Genomic DNA was isolated from leaves via CTAB-method or DNeasy Miniprotocol (QIAGEN), respectively.

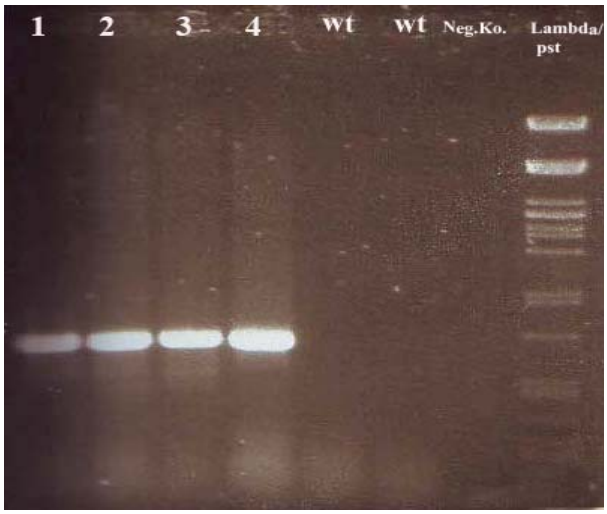
For genotyping of BX1 progenies (Fig. 62) PCR was performed under standard conditions using primer pairs: 814C11 and T-DNA, or At3g12760 for and rev, respectively. In both cases genomic DNA from wild type col-O leaves was used as a control. Using primers 814C11 and T-DNA the wild type control should show no amplification product, while using primers At3g12760 for and rev one band 2132 bp in size should appear. As a negative control ddH_2O^a was used instead of DNA.



Lane 1: 814C11/24-5-3
Lane 2: 814C11/24-5-9
Lane 3: 814C11/24-6-4
Lane 4: 814C11/24-6-7
Lane 5: 814C11/24-10-2
Lane 6: wt col-O
Lane 7: Negative Control
Lane 8: Lambda/pst marker

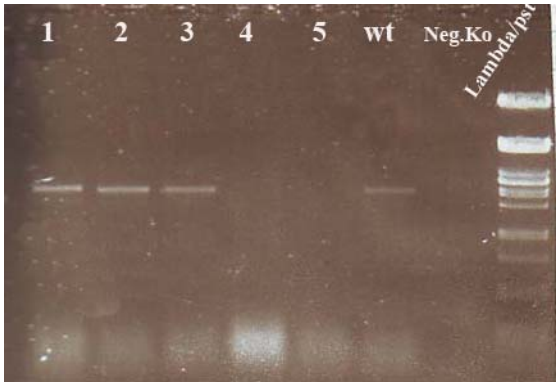
Fig. 25 Genotyping on gDNA level/ BX1a plants
BX1a plants were amplified with 814C11 and T-DNA LB primers, separated on an 1% agarose gel and visualized by ethidium bromide staining. Because of a loading mistake the wt control lane showed contamination.

As it can be seen in Fig. 25, Fig. 26 and Tab. 1 all progenies of the first backcross showed a band in proper size after amplification with the first mentioned primer pair, indicating that the T-DNA insertion is located within *AtDCN*1. Alternatively the second primer pair At3g12760 for and rev was used for genotyping on genomic DNA level, as well. If the T-DNA insertion is located within *AtDCN*1 this part would be too big to get amplified by Go Taq polymerase, using the same PCR program; if not, a band 2132 bp in size would appear on the gel. All BX1a samples, except three progenies of backcross 814C11/24-6 x wt showed an amplification product after PCR reaction, as it can be seen in Fig. 27, Fig. 28, Tab.2.



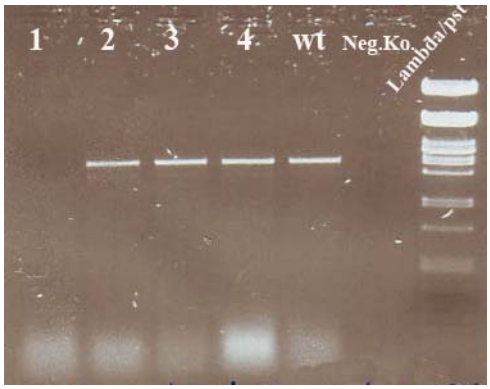
Lane 1: 814C11/24-5-6
Lane 2: 814C11/24-6-9
Lane 3: 814C11/24-10-3
Lane 4: 814C11/24-10-9
Lane 5-6: wt col-O
Lane 7: Negative Control
Lane 8: Lambda/pst marker

Fig. 26 Genotyping on gDNA level/ BX1a plants
BX1a plants were amplified with 814C11 and T-DNA LB primers, separated on an 1% agarose gel and visualized by ethidium bromide staining.



Lane 1: 814C11/24-5-3
Lane 2: 814C11/24-5-6
Lane 3: 814C11/24-5-9
Lane 4: 814C11/24-6-4
Lane 5: 814C11/24-6-7
Lane 6: wt col-O
Lane 7: Negative Control
Lane 8: Lambda/pst marker

Fig. 27 Genotyping on gDNA level/ BX1a plants
BX1a plants were amplified with At3g12760 for and rev gene specific primers, separated on an 1% agarose gel and visualized by ethidium bromide staining.



Lane 1: 814C11/24-6-9
Lane 2: 814C11/24-10-2
Lane 3: 814C11/24-10-3
Lane 4: 814C11/24-10-9
Lane 5: wt col-O
Lane 6: Negative Control
Lane 7: Lambda/pst marker

Fig. 28 Genotyping on gDNA level/ BX1a plants
BX1a plants were amplified with At3g12760 for and rev gene specific primers, separated on an 1% agarose gel and visualized by ethidium bromide staining.

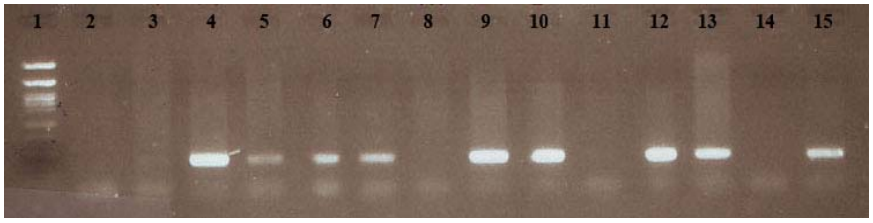
BX1a/gDNA	LB + 814C11-3'
814C11/24-5-3	positive, ~ 800 bp
814C11/24-5-6	positive, ~ 800 bp
814C11/24-5-9	positive, ~ 800 bp
814C11/24-6-4	positive, ~ 800 bp
814C11/24-6-7	positive, ~ 800 bp
814C11/24-6-9	positive, ~ 800 bp
814C11/24-10-2	positive, ~ 800 bp
814C11/24-10-3	positive, ~ 800 bp
814C11/24-10-9	positive, ~ 800 bp
Wt col-0 d	positive, ~ 800 bp
Wt col-0 6-4	Negative
Wt col-0 2-1	Negative

Tab. 1 PCR-BX1a Table/1

BX1a/gDNA	12760 3' + 5'
814C11/24-5-3	negative, ~ 2000 bp
814C11/24-5-6	negative, ~ 2000 bp
814C11/24-5-9	negative, ~ 2000 bp
814C11/24-6-4	Positive
814C11/24-6-7	Positive
814C11/24-6-9	Positive
814C11/24-10-2	negative, ~ 2000 bp
814C11/24-10-3	negative, ~ 2000 bp
814C11/24-10-9	negative, ~ 2000 bp
Wt col-0 d	positive, ~ 2000 bp

Tab. 2 PCR-BX1a Table/2

Progenies of BX2 and BX3 were checked via PCR using primer pairs GSP (gene specific primer), T-DNA LB/2 and RB, GSP/2. Genomic DNA from wild type col-O leaves was used as a control, as no band should appear using one of previous mentioned oligonucleotides, priming against parts of the gene and either the RB or LB of the insertion, respectively. As a negative control ddH_2O^a was used instead of DNA.



Lane 2: wt col-O
Lane 3: 2A/1d
Lane 4: 2B/2b
Lane 5: 2C/1a
Lane 6: 2D/2b
Lane 7: 3B/2b
Lane 8: 3D/1a
Lane 9: 4A/1c
Lane 10: 4B/2e
Lane 11: 4D/2e
Lane 12: 5B/1a
Lane 13: 5C/2d
Lane 15: 5D/2c

Fig. 29 Genotyping on gDNA level/ BX2a plants
Lane 1 shows fragments of Lambda DNA digested with *pstI*, while Lane 14 represents the negative control. The other lanes show PCR products of progenies from BX2 plants, amplified with primers GSP and T-DNA LB/2, visualized by ethidium bromide staining on a 2% agarose gel.

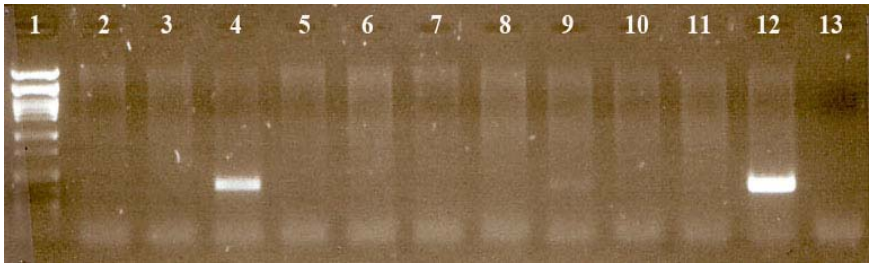


Fig. 30 Genotyping on gDNA level/ BX3 plants and their progenies
All samples represent different genotypes of BX3 plants and their progenies, while lanes 2-6 represent samples of BX3 and lanes 8-11 their progenies. Lane 1 shows a Lambda/pst marker, lane 13 the negative control, while lane 7 represents the wild type col-O control. Lane 2: 814C11/24-5-1 x wt 1e, lane 3: 814C11/24-5-1 x wt 2c, lane 4: 814C11/24-10-6 x wt c, lane 5: 814C11/24-5-5 x wt a, lane 6: 814C11/24-10-10 x wt c, lane 8: 814C11/24-5-11 x wt d, lane 9: 814C11/24-6-10 x wt B1, lane 10: 814C11/24-6-3 x wt B1, lane 11: 814C11/24-6-3 x wt C4, lane 12: 814C11/24-6-10 x wt A2. PCR was performed with primers GSP and T-DNA LB/2 and visualized by ethidium bromide staining on an 1% agarose gel.

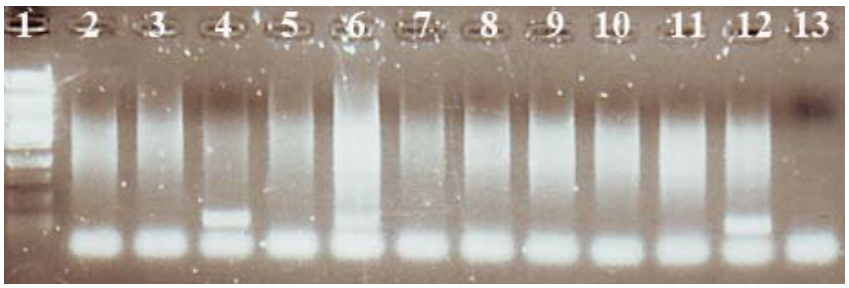


Fig. 31 Genotyping on gDNA level/ BX3 plants and their progenies
All samples represent different genotypes of BX3 plants and their progenies, while lanes 2-7 represent samples of BX3 and lanes 9-12 their progenies. Lane 1 shows Lambda/pst marker, lane 13 represents the negative control, and lane 8 represents the wild type col-O control. Lane 2: 814C11/24-5-1 x wt 2e, lane 3: 814C11/24-5-1 x wt 1c, lane 4: 814C11/24-10-6 x wt c, lane 5: 814C11/24-5-5 x wt a, lane 6: 814C11/24-10-10 x wt e, lane 8: 814C11/24-5-11 x wt d, lane 9: 814C11/24-6-10 x wt B1, lane 10: 814C11/24-6-3 x wt B1, lane 11: 814C11/24-6-3 x wt C4, lane 12: 814C11/24-6-10 x wt A2. PCR was performed with primers GSP/2, RB and visualized by ethidium bromide staining on an 1% agarose gel.

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Genotyping of BX2, BX3 and their progenies was repeated twice with each primer pair (Fig. of second approach not shown) as it can be seen in Tab.3. Several progenies of BX2 (2B/2b, 2C/1a, 2D/2b, 3B/2b, 4A/1c, 5B/1a, 5C/2d) as well as one BX3 (814C11/24-6-10 x wt A2) plant revealed positive results in every genotyping approach checked by both primer pairs shown in Fig. 29, Fig. 30, Fig. 31, Fig. 32 and Tab.3

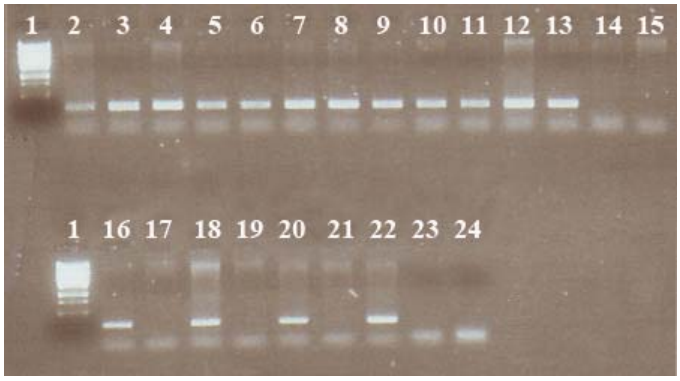


Fig. 32 Genotyping on gDNA level/ BXn and their progenies
Lane 1 represents a Lambda/pst marker, Lane 14 a wild type control, while lane 24 represents the negative control, lane 2: 2A/1d, lane 3: 2B/2b, lane 4: 2C/1a, lane 5: 2D/2b, lane 6: 3B/2b, lane 7: 3D/1a, lane 8: 4A/1c, lane 9: 4B/2e, lane 10: 4D/2e, lane 11: 5B/1a, lane 12:5C/2d, lane 13: 5D/2c, lane 15: 814C11/24-5-1 x wt 1e, lane 16: 814C11/24-5-1 x wt 2e, lane 17: 814C11/2410-6 x wt c, lane 18: 814C11/24-5-5 x wt a, lane 19: 814C11/24-10-10 x wt e, lane 20: 814C11/24-5-11 x wt d, lane 21: 814C11/24-6-10 x wt B1, lane 22: 814C11/24-6-3 x wt B1 and lane 23: 814C11/24-6-3 x wt C4. Lanes 2-12 represent samples from BX2a, lanes 15-20 BX3 and lanes 21-23 their progenies. PCR was performed with primers GSP/2, RB and visualized by ethidium bromide staining on an 1% agarose gel.

Genotyp	LB/GSP (382bp)	RB/GSP (552bp)
Wt/1A/1b	- ; -	-
Wt/1B/1d		-
BX2a/2A/1d	+ ; (~)	- ; +
BX2a/2A/2b		+
BX2a/2B/2b	+ ; +	+
BX2a/2C/1a	+ ; +	+
BX2a/2D/2b	+ ; +	+ ; (~)
BX2a/3B/2b	+ ; +	+ ; +
BX2a/3D/1a	- ; +	+ ; +
BX2a/4A/1c	+	+
BX2a/4B/2e	+ ; +	- ; +
BX2a/4D/2e	- ; +	(~); +

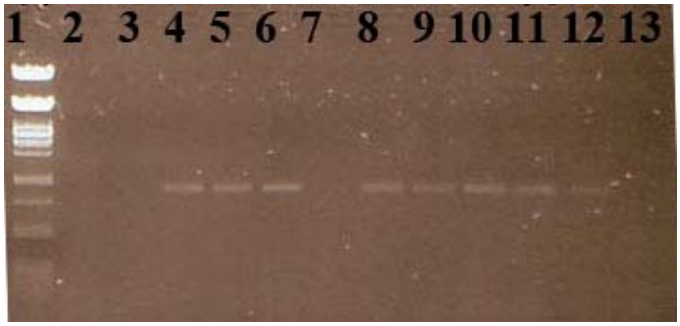
BX2a/5B/1a	+ ; +	+
BX2a/5C/2d	+ ; +	(~);+
BX2a/5D/2c	+ ; +	- ; +
BX3/5-1xe/1/e	- ; ~	- ; -
BX3/5-1xe/2/e	- ; -	- ; +
BX3/10-6xe/c	+ ; +	+ ; -
BX3/5-5xe/a	- ; -	- ; +
BX3/10-10xj/e	+	+ ; -
BX3/5-11xh/d	- ; -	- ; +
BX3a wt_0/f/2		- ; -
BX3a wt_0/f/3	- ; +	
BX3a/6-10xh/B1	(~);+	- ; -
BX3a/6-3xj/B1	- ; -	- ; +
BX3a/6-3xj/C4	- ; +	- ; -
BX3a/6-10xh/A2	+	+

Tab. 3 Genotyping/ BX2a/ BX3 and their progenies
(~) denotes weak signals; - denotes no signal, + denotes signal.

7.4. Expression analysis via RT-PCR of all backcrosses and their progenies

To check the expression analysis of different backcrosses (BXn) and their progenies (Bxna) total RNA was isolated from leaves or seedlings, respectively using RNeasy Miniprotocol (QIAGEN) and translated into cDNA using AMV-RT after DNase I digestion, as described in Materials and Methods.

If the T-DNA insertion disrupts the desired gene *AtDCN*1, no transcript is expected to be present in the plant, hence no band should appear on the gel after amplification using gene specific primers. This could be approved for two BX1a (814C11/24-6-4_{23}, 814C11/24-6-4_{25}) and two BX2a samples (2B/3c, 4B/2d), as well as for one BX3a sample 814C11/24-6-3 x wt A4 (Fig. 33, Fig. 34, Tab. 4). As a control the efficiency of cDNA synthesis was checked using 18s primers (Fig. 69).



Lane 1: Lambda/pst marker
Lane 2: 814C11/24-6-4_{23}
Lane 3: 814C11/24-6-4_{25}
Lane 4: 814C11/24-6-7_{23}
Lane 5: 814C11/24-6-7_{25}
Lane 6: 2A/1
Lane 7: 2B/3
Lane 8: 2D/3
Lane 9: 4A/2
Lane 10: 4B/3
Lane 11: wt col-O
Lane 12: wt col-O
Lane 13: Negative control

Fig. 33 *AtDCN*1 expression analysis/ BX1a/ BX2a plants
Material was isolated from approximately two weeks old BX1a and BX2a seedlings and checked via PCR on cDNA level, as described in Materials and Methods. PCR products amplified with At3g12760 gene specific primers were separated on an 1% agarose gel and visualized by ethidium bromide staining.

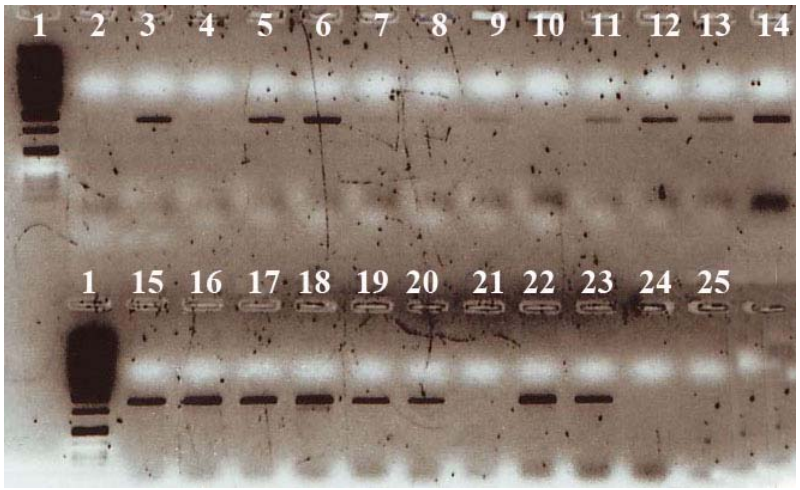


Fig. 34 *AtDCN*1 expression analysis/ BX2a/ BX3 and their progenies
For RNA isolation of BX2a, BX3 and BX3a plants, material was isolated from leaves and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were separated on an 1% agarose gel and visualized by ethidium bromide staining.

Lane 1: Lambda/pst marker
Lane 2: empty
Lane 3: 2A/2e
Lane 4: 2B/3c
Lane 5: 2C/3d
Lane 6: 2D/3c
Lane 7: 3A/2a
Lane 8: x
Lane 9: 4A/1d
Lane 10: 4B/2d
Lane 11: 4D/2b
Lane 12: 5B/2b
Lane 13: 5C/2e
Lane 14: 5D/1d
Lane 15: wt col-O
Lane 16: 814C11/24-5-11 x wt b
Lane 17: 814C11/24-5-1 x wt 1a
Lane 18: 814C11/24-5-1 x wt 2a
Lane 19: 814C11/24- 10-10 x wt b
Lane 20: 814C11/24-10-6 x wt a
Lane 21: 814C11/24-6-10 x wt A4
Lane 22: 814C11/24-6-3 x wt B3
Lane 23: 814C11/24-6-10 x wt B4
Lane 24: 814C11/24-6-3 x wt C2
Lane 25: Negaitve Control

Genotyp	Anfang primer	Ende primer	*AtDCN*1 GSP (~900bp)	*18S primer*
Wt/1B/1e	++	+	+	+ ; +
BX2a/2A/2e	++	++	+ ; +	+ ; +
BX2a/2B/3c	++	+	- ; -	+ ; +
BX2a/2C/3d	++	+	+ ; +	+ ; +
BX2a/2D/3c	++	++	++ ; +	+ ; +
BX2a/3A/2a	++	++	+(~) ; -	+ ; +
BX2a/3B/2c	-	-		
BX2a/4A/1d	-	-	+(~) ; -	+
BX2a/4B/2d	+	+	-	+
BX2a/4D/2b	+(~)	+	+ ;+ (~)	+
BX2a/5B/2b	+(~)	+	+ ;+ (~)	+
BX2a/5C/2e	+(~)	+(~)	+ ;+ (~)	+
BX2a/5D/1d	+(~)	+(~)	+ ;+ (~)	+
BX3 wt col-O	+(~)	++	+ ; ++	+
BX3/5-1xe/1a	+(~)	-	+ ;+ (~)	+
BX3/5-1xe/2a	+(~)	-	+ ;+ (~)	+
BX3/10-6xe/a	+(~)	+(~)	+ ;+ (~)	+
BX3/10-10xj/b	+	+	+ ; +	+
BX3/5-11xh/b	+	+	+ ; -	+
BX3a/6-10xh/B4	+	+	+ ; +	+
BX3a/6-3xj/B3	+	+	+ ; +	+
BX3a/6-3xj/C2	+(~)	+(~)	- ; -	+
BX3a/6-10xh/A4	-	-	- ; -	+

Tab. 4 Expression analysis/ BX2a/ BX3 and their progenies

(~) denotes weak signals; - denotes no signal, + denotes signal, ++ denotes very strong signal, while this sign ; separates two independent approaches.

7.4.1 Expression analysis of two further genes (At2g28540, At3g12770)

GABI-Kat mentioned a possible second insertion to be located in At2g28540 or in At3g12770, respectively. Results from amplification with respective gene specific primers on cDNA level revealed the presence of proper transcripts for both genes in all checked BX1a and BX2a samples, as well as for the wild type col-O control (Fig. 35, Fig. 36).

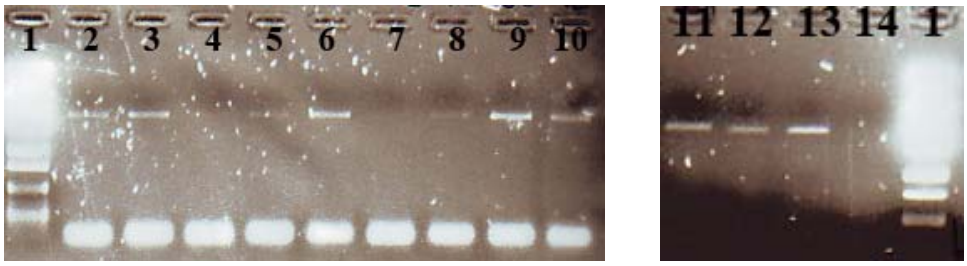


Fig. 35 At2g28540 Expression analysis/ BX1a/ BX2a plants
RNA was isolated from approximately two weeks old seedlings and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were separated on an 1% agarose gel and visualized by ethidium bromide staining.

Lane 1: Lambda/pst marker
Lane 2: 814C11/24-6-4_{23}
Lane 3: 814C11/24-6-4_{25}
Lane 4: 814C11/24-6-7_{23}
Lane 5: 814C11/24-6-7_{25}
Lane 6: 2A/1
Lane 7: 2B/3
Lane 8: 2D/3
Lane 9: 4A/2
Lane 10: 4B/3
Lane 11: wt col-O
Lane 12: wt col-O
Lane 13: wt col-O
Lane 14: Negative Control

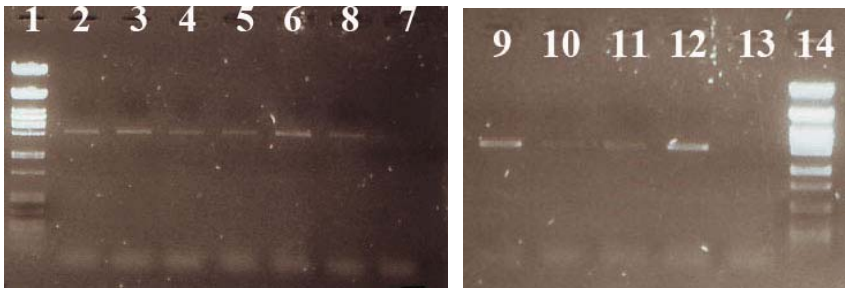


Fig. 36 At3g12770 Expression analysis/ BX1a/ BX2a plants
RNA was isolated from approximately two weeks old seedlings and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were separated on an 1% agarose gel and visualized by ethidium bromide staining.

Lane 1: Lambda/pst marker
Lane 2: 814C11/24-6-4_{23}
Lane 3: 814C11/24-6-4_{25}
Lane 4: 814C11/24-6-7_{23}
Lane 5: 814C11/24-6-7_{25}
Lane 6: 2A/1
Lane 7: 2B/3
Lane 8: 2D/3
Lane 9: 4A/2
Lane 10: 4B/3
Lane 11: wt col-O
Lane 12: wt col-O
Lane 13: wt col-O
Lane 14: Negative Control

7.5. Amplification and purification of DNA and RNA blot probes

The probes for radioactive labelling and further hybridization of DNA blots were amplified via PCR from BX1a genomic DNA and purified via Wizard SV gel and PCR purification system. One probe is 433 bp in size and its binding site is located approximately in the middle of the T-DNA insertion, between the flanking left (LB) and the right boarder (RB) sequences. In spite of everything this probe was referred as RB probe (Fig. 37). Another probe was designed to bind to the LB of the T-DNA insertion, is 476 bp in size and was referred as LB probe (second LB probe) (Fig. 38).

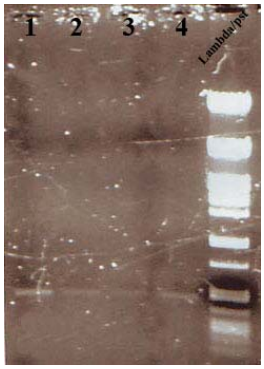


Fig. 37 Purified Southern blot probe/ RB

Fig. 38 Purified Southern blot probe/ LB

After amplification via PCR, the probe was purified and visualized by ethidium bromide staining on an 0,8% agarose gel. Fig. 29: Lanes 1-4 represent different genotypes of BX1a samples, while probe in lane 1 was used for further hybridization of Southern blots. Fig. 30: Lanes 1-2 represent different genotypes of BX1a samples, while probe in lane 1 was used for further re-hybridization of the blotted membranes.

As a probe for Northern blotting, cDNA synthesized from wild type col-O leaves amplified with At3g12760 gene specific primers and purified via Wizard SV gel and PCR purification system was used (Fig. 39).

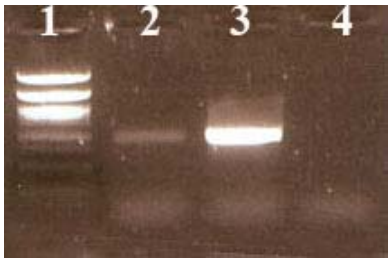


Fig. 39 Northern Blot probe before purification

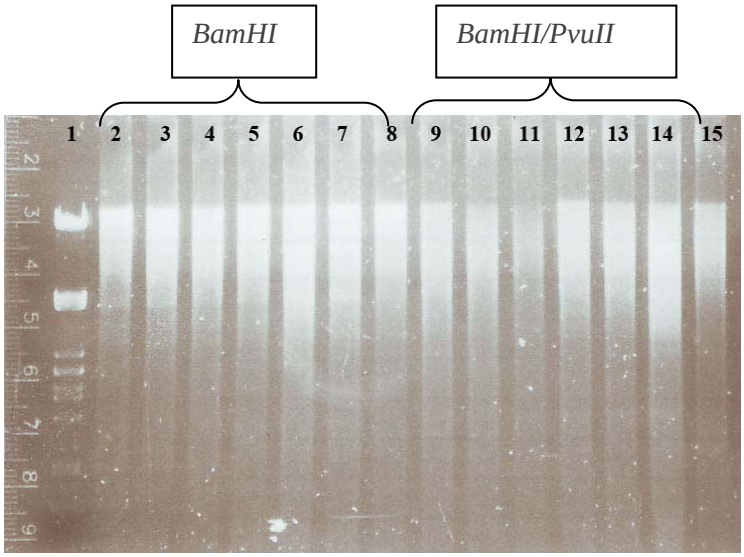
After amplification of wild type col-O cDNA with At3g12760 gene specific primers, the PCR product was visualized by ethidium bromide staining on an 0,8% agarose gel. Lane 1 represents length marker Lambda/pst, lanes 2 and 3 show one band 1022bp in size amplified from wild type cDNA and lane 4 represents the negative control.

7.6.Characterization of different backcrosses and their progenies via Southern blot analysis

To determine the number of T-DNA insertions after backcrossing Southern blot analysis was performed as described in Materials and Methods. The RB probe was designed for restriction with *BamHI;* the enzyme cuts once in front of the probe binding site but not elsewhere in the insertion. If because of backcrossing of a mutant plant to a wild type background one of the two initially determined T-DNA insertions was crossed out, we would expect one band with unknown size on the blot when cut with *BamHI*. The second enzyme which was used for restriction of gDNA was *PvuII,* which cuts once within in the RB probe binding site. As a result for *BamHI/ PvuII* double digestion we would expect two smaller bands on the blot whose sum is the size of the big band appearing when cut with *BamHI*.

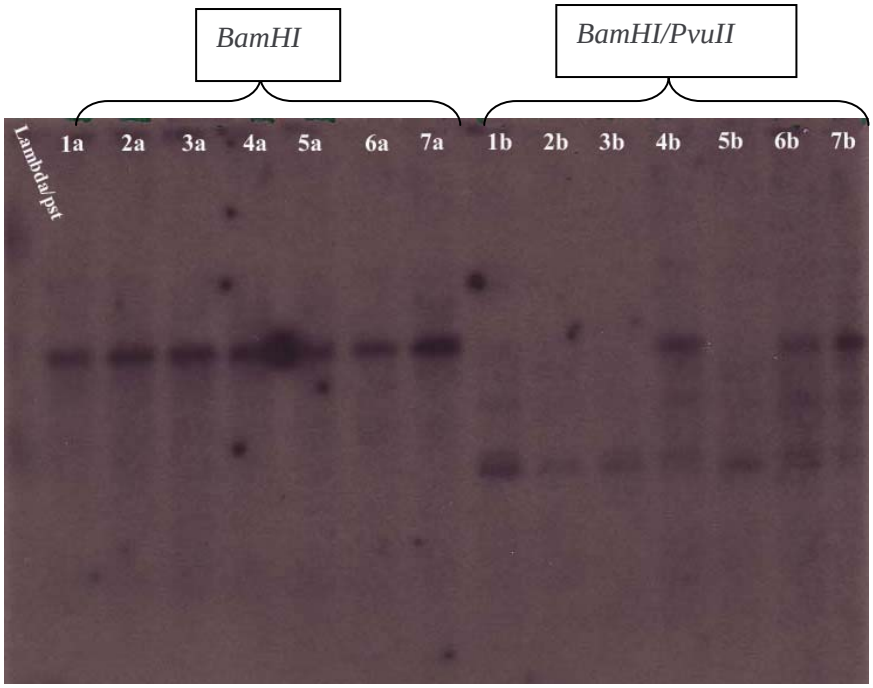
After stripping the membrane, the same was hybridized with another probe against the LB of the T-DNA insertion. When cut with *BamHI* the result was expected to be same as previous, one band with unknown size on the blot if the backcross was successful. Using this LB probe would not change the expected result when performing *BamHI/ PvuII* double digestion as *PvuII* cuts outside of the LB probe binding site

As it can be seen in Fig. 40 – Fig. 49 one up to five insertions were detected in different backcrosses either probing against the RB or LB, using different restriction enzymes which awaits further explanation.



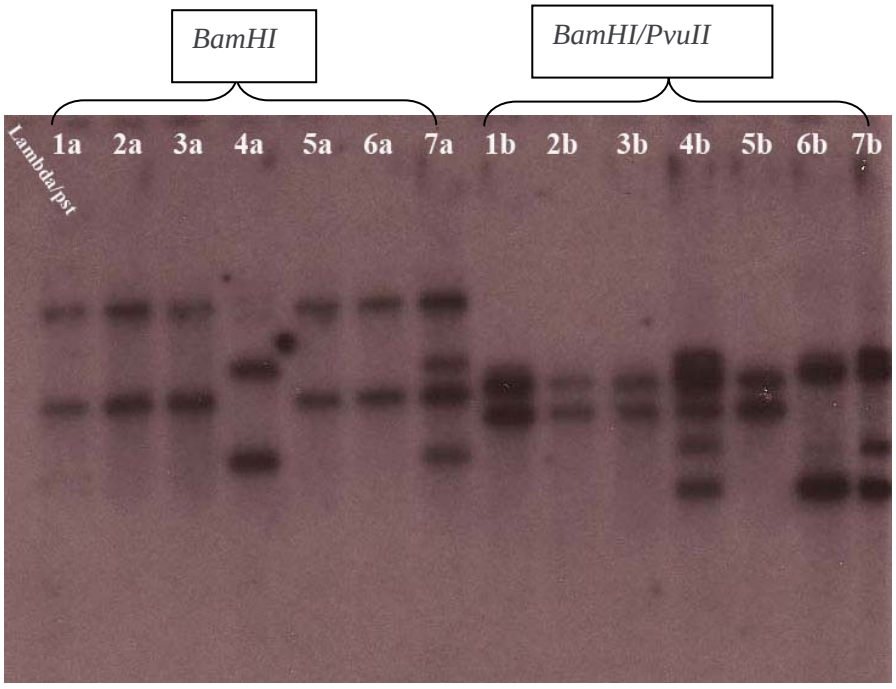
Lane 1: Lambda/pst marker
Lane 2: 2A/2b Lane 9: 2A/2c
Lane 3: 2D/2c Lane 10: 2D/2e
Lane 4: 2D73a Lane 11: 2D/3b
Lane 5: 3A/1a Lane 12: 3A/1e
Lane 6: 3B/2a Lane 13: 3B/2e
Lane 7: 3D/1b Lane 14: 3D/1e
Lane 8: 3D/3a Lane 15: 3D/3b

Fig. 40 Restriction of gDNA with *BamHI*, *BamHI/ PvuII;* Southern blot/ BX2a plants
8-10 µg gDNA isolated from BX2a leaves were cut with *BamHI* (lanes 2-8) and *BamHI/ PvuII* (lane 9-15),separated on an 0,8% agarose gel and visualized by ethidium bromide staining.



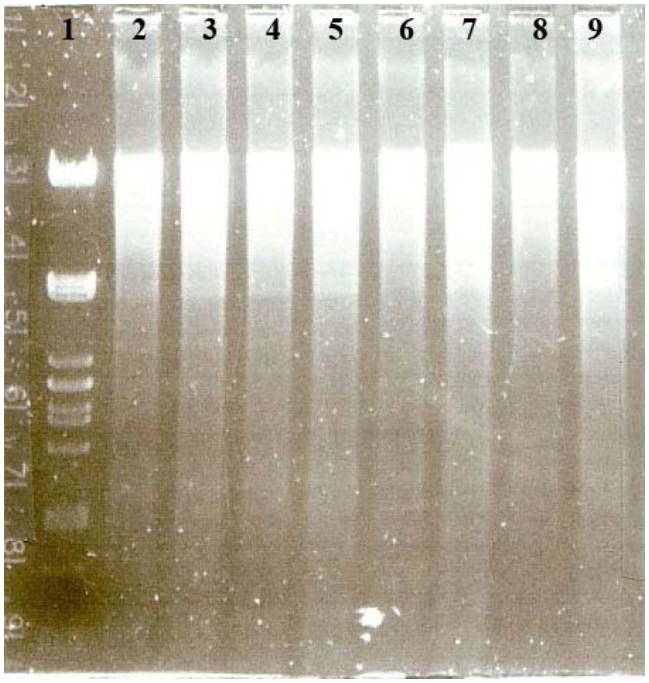
1a: 2A/2b 1b: 2A/2c
2a: 2D/2c 2b: 2D/2e
3a: 2D73a 3b: 2D/3b
4a: 3A/1a 4b: 3A/1e
5a: 3B/2a 5b: 3B/2e
6a: 3D/1b 6b: 3D/1e
7a: 3D/3a 7b: 3D/3b

Fig. 41 Southern blot probed against the RB/ BX2a plants
8-10 µg gDNA isolated from BX2a leaves were once digested with *BamHI* (1a-7a) and once with *BamHI/ PvuII* (1b-7b). Each lane with same number but different letter e.g., 1a and 1b represents the same genotype. The membrane was hybridized o/n at 65°C with an [α-^{32}P]dCTP-labelled probe against the RB of the T-DNA insertion.



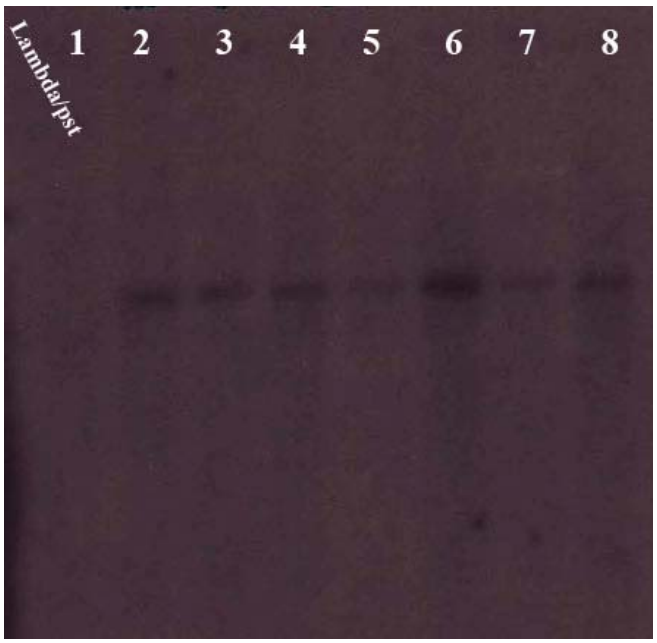
1a: 2A/2b 1b: 2A/2c
2a: 2D/2c 2b: 2D/2e
3a: 2D73a 3b: 2D/3b
4a: 3A/1a 4b: 3A/1e
5a: 3B/2a 5b: 3B/2e
6a: 3D/1b 6b: 3D/1e
7a: 3D/3a 7b: 3D/3b

Fig. 42 Strip/ Southern blot probed against LB/ BX2a plants
8-10 µg gDNA isolated from BX2a leaves were once digested with *BamHI* (1a-7a) and once with *BamHI/PvuII* (1b-7b), while *PvuI* cuts once within in the RB probe binding site Two lanes with the same number but different letter e.g., 1a and 1b represents the same genotype. The membrane was stripped and re-hybridized o/n at 65°C with an [α-^{32}P]dCTP-labelled probe against the LB of the T-DNA insertion.



Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: 4A/1a
Lane 4: 4A/2e
Lane 5: 4B/2c
Lane 6: 4D/2d
Lane 7: 5C/2b
Lane 8: 5D/2e
Lane 9: 814C11/24-6-10 x wt A1

Fig. 43 Restriction of gDNA with *BamHI;* Southern blot/ BX2a / BX3a plants
8-10 µg gDNA isolated from BX2a and BX3a leaves were cut with *BamHI* (lanes 2-9), separated on an 0,8% agarose gel and visualized by ethidium bromide staining.

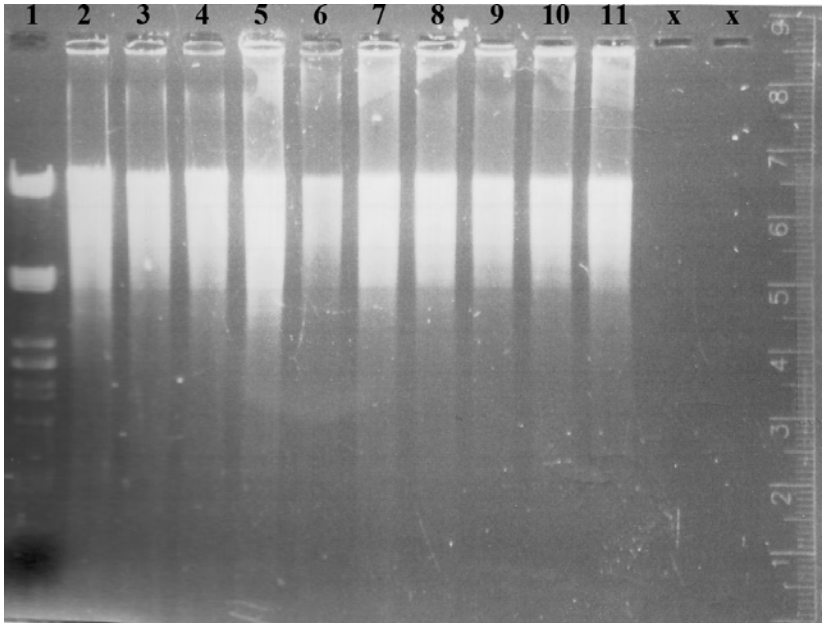


Lane 1: wt col-O
Lane 2: 4A/1a
Lane 3: 4A/2e
Lane 4: 4B/2c
Lane 5: 4D/2d
Lane 6: 5C/2b
Lane 7: 5D/2e
Lane 8 814C11/24-6-10 x wt A1

Fig. 44 Southern blot probed against the RB/ BX2a/ BX3a plants
Genomic DNA was isolated from leaves and digested o/n with *BamHI*. Lanes 2-7 represent samples from BX2a plants, while lane 8 represents one progeny of BX3. The membrane was hybridized o/n at 65°C with a an [α-^{32}P]dCTP-labelled probe against the RB of the T-DNA insertion.

Lane 1: wt col-O
Lane 2: 4A/1a
Lane 3: 4A/2e
Lane 4: 4B/2c
Lane 5: 4D/2d
Lane 6: 5C/2b
Lane 7: 5D/2e
Lane 8 814C11/24-6-10 x wt A1

Fig. 45 Strip/Southern blot probed against LB/ BX2a/ BX3a plants
Genomic DNA was isolated from leaves and digested o/n with *BamHI*. Lanes 2-7 represent samples from BX2a plants, while lane 8 represents one progeny of BX3. The membrane was stripped and re-hybridized o/n at 65°C with an [α-^{32}P]dCTP-labelled probe against the LB of the T-DNA insertion.



Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: 814C11/24-5-1 x wt 2c
Lane 4: 814C11/24-5-1 x wt 3d
Lane 5: 814C11/24-10-6 x wt b
Lane 6: 814C11/24-5-11 x wt c
Lane 7: wt col-O
Lane 8: 814C11/24-6-10 x wt B2
Lane 9: 814C11/24-6-3 x wt B2
Lane 10: 814C11/24-6-3 x wt C1
Lane 11: 814C11/24-6-10 x wt A1

Fig. 46 Restriction of gDNA with *BamHI*, Southern blot/ BX3/ BX3a plants
8-10 µg gDNA isolated from BX3 and BX3a leaves were cut with *BamHI* (lanes 2-11), separated on an 0,8% agarose gel and visualized by ethidium bromide staining. Lanes 3-6 represent different samples of BX3, while lanes 8-11 represent different samples from their progenies (BX3a).

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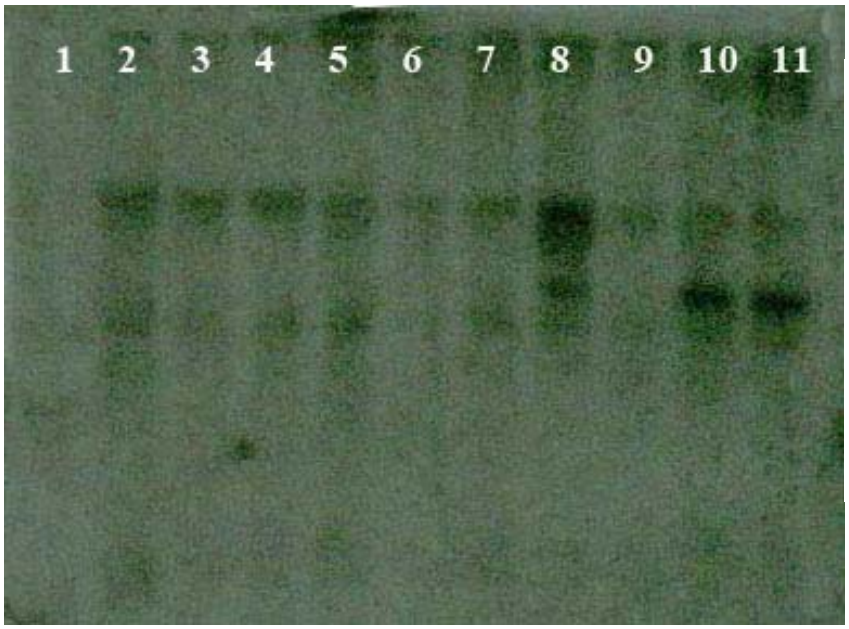
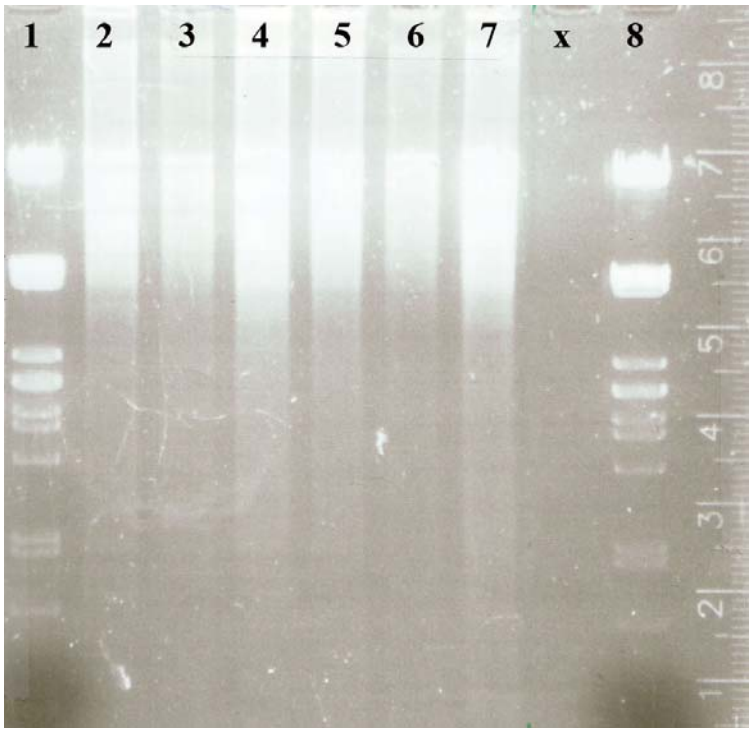


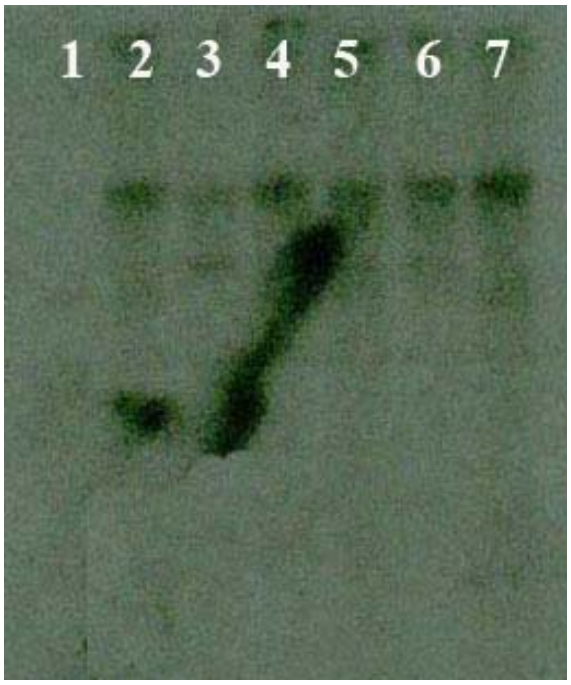
Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: 814C11/24-5-1 x wt 2c
Lane 4: 814C11/24-5-1 x wt 3d
Lane 5: 814C11/24-10-6 x wt b
Lane 6: 814C11/24-5-11 x wt c
Lane 7: wt col-O
Lane 8: 814C11/24-6-10 x wt B2
Lane 9: 814C11/24-6-3 x wt B2
Lane 10: 814C11/24-6-3 x wt C1
Lane 11: 814C11/24-6-10 x wt A1

Fig. 47 Southern blot probed against the RB/ BX3/ BX3a plants
Genomic DNA was isolated from leaves and digested o/n with *BamHI*. Lanes 3-6 represent different samples of BX3, while lanes 8-11 represent different samples from their progenies (BX3a). The membrane was hybridized o/n at 65°C with an [α-^{32}P]dCTP-labelled probe against the RB of the T-DNA insertion.



Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: 2B/2c
Lane 4: 2C/1c
Lane 5: 4A/2b
Lane 6: 5B/1c
Lane 7: 814C11/24-10-10 x wt c
Lane 8: Lambda/pst marker

Fig. 48 Restriction of gDNA with *BamHI*, Southern blot/ BX2a/ BX3 plants
8-10 µg gDNA isolated from BX2a and BX3 leaves were cut with *BamHI* (lanes 2-7), separated on an 0,8% agarose gel and visualized by ethidium bromide staining. Lanes 3-5 represent different samples of BX2a, while lane 7 represents one sample of BX3.
.



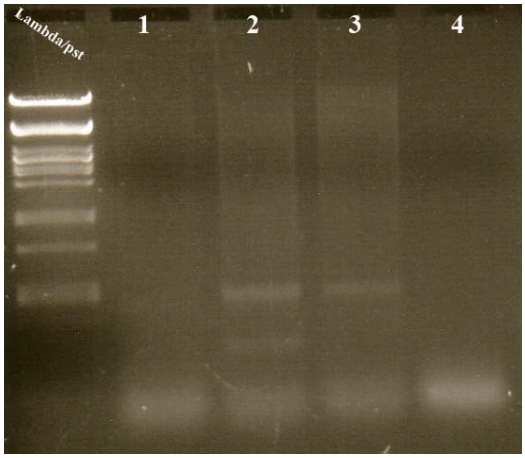
Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: 2B/2c
Lane 4: 2C/1c
Lane 5: 4A/2b
Lane 6: 5B/1c
Lane 7: 814C11/24-10-10 x wt c
Lane 8: Lambda/pst marker

Fig. 49 Southern blot probed against the RB/ BX2a/ BX3 plants
Genomic DNA was isolated from leaves and digested o/n with *BamHI*. Lanes 3-5 represent different samples of BX2a, while lane 7 represents one sample of BX3. The membrane was hybridized o/n at 65°C with an [α-^{32}P]dCTP-labelled probe against the RB of the T-DNA insertion.

7.7. Check backcrossed plants for multiple insertions within *AtDCN*1

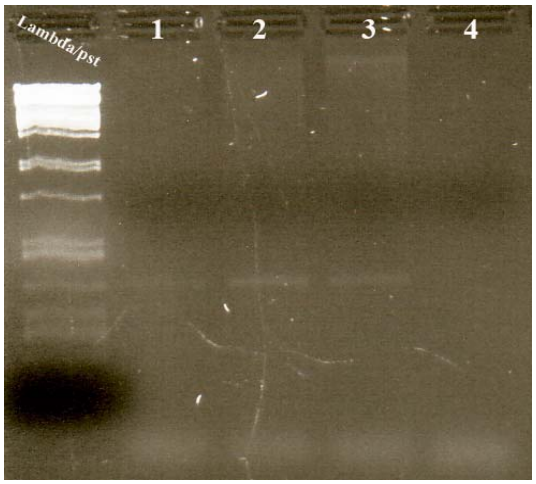
The possible presence of two T-DNA insertions located RB to RB within *AtDCN*1 was checked via PCR using two different primer pairs, amplifying 400 or 900 bp around adjacent RBs, respectively (Fig. 11).

Checked BX1a and BX2a plants showed one band in proper size after amplification with primers RB 400 while for one sample an additional second band appeared (Fig. 50). PCR with primers RB 900 revealed proper signals for backcrossed plants but unfortunately the wild type control showed a very weak band as well, which could not be impeded in several different PCR attempts (Fig. 51).



Lane 1: wt col-O
Lane 2: 4A/1c
Lane 3: 814C11/24-5-5 x wt a
Lane 4: Negative Control

Fig. 50 PCR on gDNA level/ RB 400 primers
Genomic DNA was isolated from leaves, using DNeasy Miniprotocol (QIAGEN) and amplified with primer pair RB 400. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. Lane 2 represents a sample from BX2a, while lane 3 shows a BX3 sample.



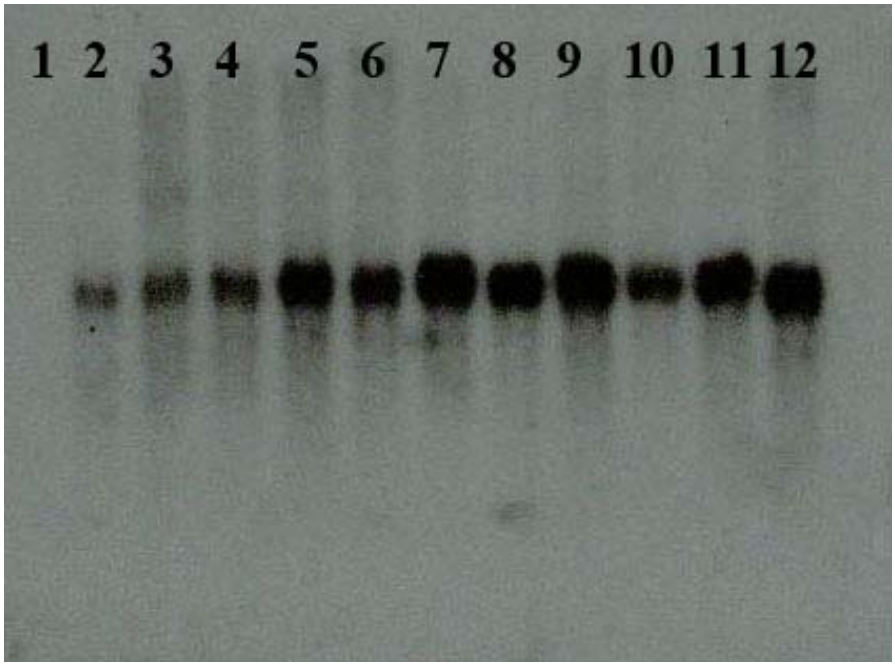
Lane 1: wt col-O
Lane 2: 4A/1c
Lane 3: 814C11/24-5-5 x wt a
Lane 4: Negative Control

Fig. 51 PCR on gDNA level/ RB 900 primers
Genomic DNA was isolated from leaves, using DNeasy Miniprotocol (QIAGEN) and amplified with primer pair RB 900. PCR products were separated on an 1% agarose gel and visualized by ethidium bromide staining. Lane 2 represents a sample from BX2a, while lane 3 shows a BX3 samples.

7.8. Expression analysis via Northern blotting of all BX and their progenies

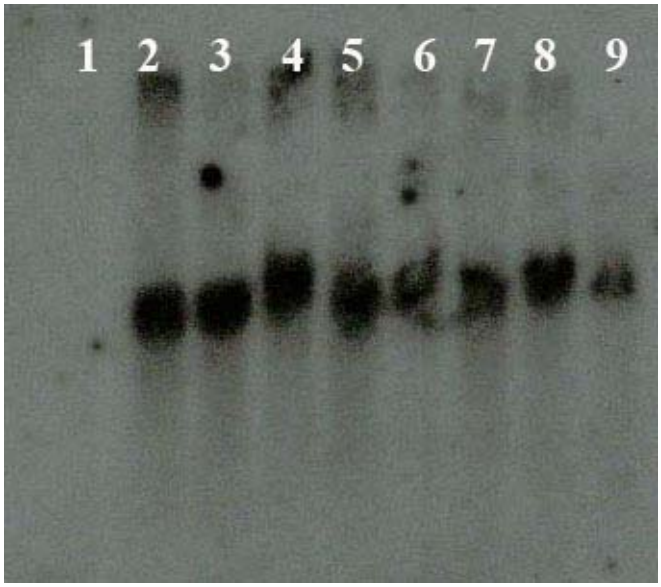
Expression analysis of all different backcrosses and their progenies was performed on cDNA level via PCR, as well as on RNA level via Northern blotting. Both expression approaches were expected to show the same result; no *AtDCN*1 transcript should be present if the T-DNA is inserted in the desired gene and if the plant is homozygous for this mutation. For all Northern blots cDNA amplified from wild type col-O leaves using At3g12760 gene specific primers, labelled with [α-^{32}P]dCTP was used as a probe, while RNA isolated from wild type leaves was used as a control.

Unfortunately for every wild type control, as well as mutant plants weak up to strong signals in proper size were detected (Fig. 52 – Fig. 55).



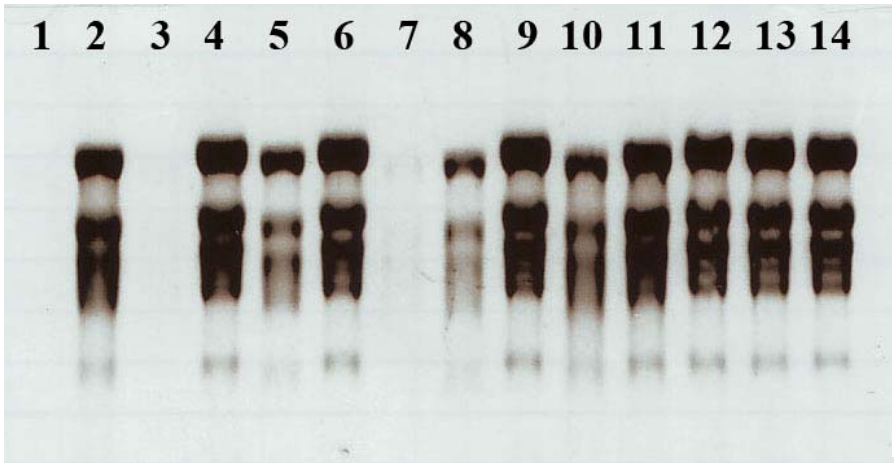
Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: 2A/2a
Lane 4: 2B/3d
Lane 5: 2D/2d
Lane 6: 2C/3a
Lane 7: 3A/1b
Lane 8: 3B/3d
Lane 9: 3D/2c
Lane 10: 4A/3c
Lane 11: 4B/2b
Lane 12: 5C/2b

Fig. 52 Northern blot/ BX2a plants
Total RNA was isolated from leaves using RNeasy Miniprotocol (QIAGEN) , brought to a concentration of 10-15 µg/6 µL and was separated on an 1,5% agarose gel. Lanes 3-12 represent samples from BX2a plants hybridized o/n at 65°C with an [α-^{32}P]dCTP-labelled probe.



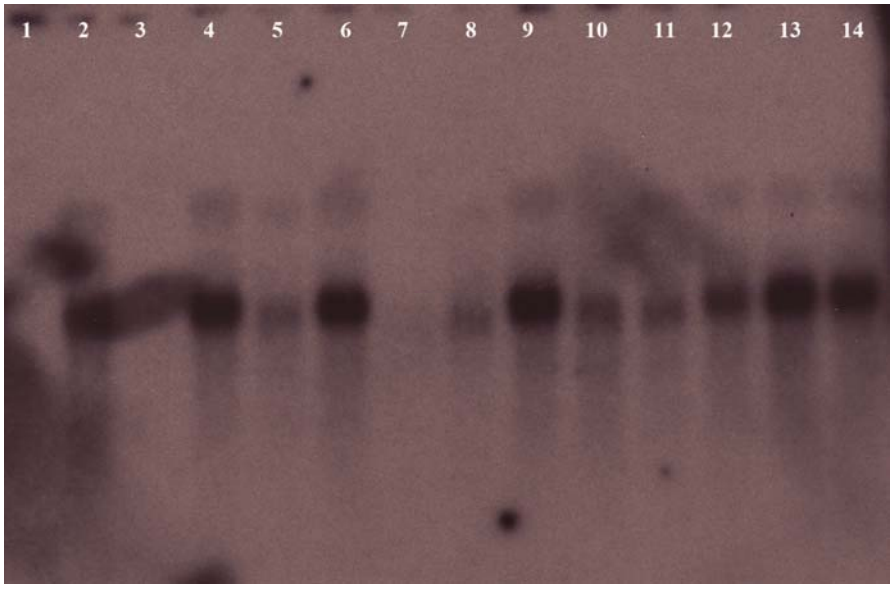
Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: 814C11/24-5-1- x wt 1b
Lane 4: 814C11/24-5-1 x wt 2b
Lane 5: 814C11/24-5-11 x wt e
Lane 6: 814C11/24-6-10 x wt A3
Lane 7: 814C11/24-6-10 x wt B5
Lane 8: 814C11/24-6-3 x wt B4
Lane 9: 814C11/24-6-3 x wt C4

Fig. 53 Northern blot/ BX3 and their progenies
Total RNA was isolated from leaves using RNeasy Miniprotocol (QIAGEN), brought to a concentration of 10-15 µg/6 µL and was separated on an 1,5% agarose gel. Lanes 3- 5 represent samples of BX3 while lanes 6-9 represent samples of BX3a plants. All samples were hybridized o/n at 65°C with an [α-^{32}P]dCTP-labelled probe.



Lane 1: Lambda/pst marker
Lane 2: 2B/3a
Lane 3: 3A/2b
Lane 4: 4A/1b
Lane 5: 4B/3e
Lane 6: wt col-O
Lane 7: 814C11/24-5-1 x wt 2d
Lane 8: 814C11/24-5-1 x wt 1d
Lane 9: 814C11/24-5-11 x wt a
Lane 10: 814C11/24-10-10 x wt d
Lane 11: 814C11/24-6-10 x wt A5
Lane 12: 814C11/24-6-10 x wt B3
Lane 13: 814C11/24-6-3 x wt B5
Lane 14: 814C11/24-6-3 x wt C5

Fig. 54 RNA before Northern blotting/ repetition of interesting backcrosses
Total RNA was isolated from leaves using RNeasy Miniprotocol (QIAGEN), brought to a concentration of 10-15 µg/6 µL and was separated on an 1,5% agarose gel. Lanes 2-5 represent samples from BX2a plants, lanes 7-10 samples from BX3 and lanes 11-14 genotypes from BX3a plants. For following samples less concentration was applied for Northern Blotting : Lane 3: 3A/2b (0,39 µg/6 µL), lane 7: 814C11/24-5-1 x wt 2d (0,49 µg/6 µL) and lane 10: 814C11-24-10-10 x wt d (9,28 µg/6 µL)



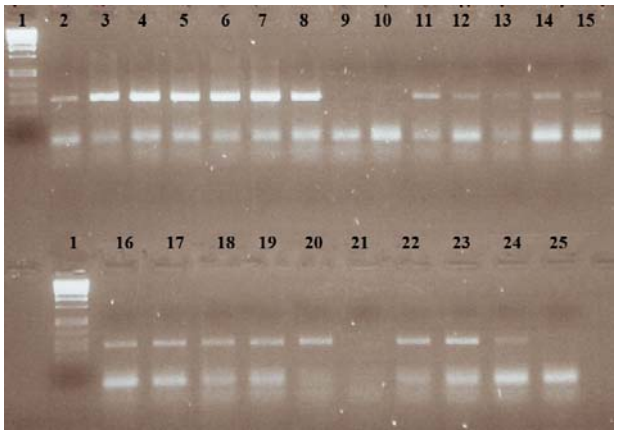
Lane 1: Lambda/pst marker
Lane 2: 2B/3a
Lane 3: 3A/2b
Lane 4: 4A/1b
Lane 5: 4B/3e
Lane 6: wt col-O
Lane 7: 814C11/24-5-1 x wt 2d
Lane 8: 814C11/24-5-1 x wt 1d
Lane 9: 814C11/24-5-11 x wt a
Lane 10: 814C11/24-10-10 x wt d
Lane 11: 814C11/24-6-10 x wt A5
Lane 12: 814C11/24-6-10 x wt B3
Lane 13: 814C11/24-6-3 x wt B5
Lane 14: 814C11/24-6-3 x wt C5

Fig. 55 Northern blot/ repetition of interesting backcrosses
Total RNA was isolated from leaves using RNeasy Miniprotocol (QIAGEN), brought to a concentration of 10-15 µg/6 µL and was separated on an 1,5% agarose gel (Fig. 51). Lanes 2-5 represent samples from BX2a plants, lanes 7-10 samples from BX3 and lanes 11-14 samples from BX3a plants. For following samples less concentration was applied for Northern Blotting (Fig. 45): Lane 3: 3A/2b (0,39 µg/6 µL), Lane 7: 814C11/24-5-1 x wt 2d (0,49 µg/6 µL) and Lane 10: 814C11-24-10-10 x wt d (9,28 µg/6 µL). All samples were hybridized o/n at 65°C with an [α-^{32}P]dCTP-labelled probe.

7.9. Check the expression of truncated versions of an *AtDCN*1 transcript

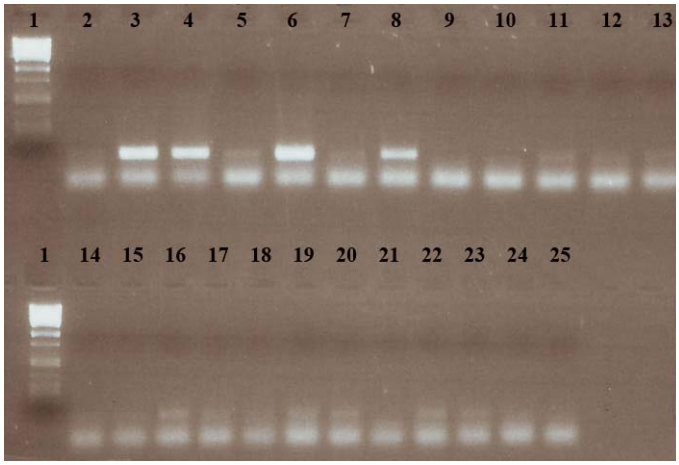
To check if truncated versions of an *AtDCN*1 transcript were expressed in backcrossed plants, PRC was performed using primers amplifying the 3'- or the 5' end of the gene, respectively.

Almost all checked plants showed the presence of either the 3'- or the 5'- end of the *AtDCN*1 transcript, amplified with either anfang (Fig. 56) or ende primers (Fig. 57).



Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: wt col-O
Lane 4: 2A/2e
Lane 5: 2B/3c
Lane 6: 2D/3c
Lane 7: 2C/3d
Lane 8: 3A/2a
Lane 9: 3B/2e
Lane 10: 4A/1d
Lane 11: 4B/2d
Lane 12: 4D/2b
Lane 13: 5B/2b
Lane 14: 5C/2e
Lane 15: 5D/1d
Lane 16: 814C11/24-5-1 x wt 1a
Lane 17: 814C11/24-5-1 x wt 2a
Lane 18: 814C11/24-10-6 x wt a
Lane 19: 814C11/24- 10-10 x wt b
Lane 20: 814C11/24-5-11 x wt b
Lane 21: 814C11/24-6-10 x wt A4
Lane 22: 814C11/24-6-3 x wt B3
Lane 23: 814C11/24-6-10 x wt B4
Lane 24: 814C11/24-6-3 x wt C2
Lane 25: Negaitve Control

Fig. 56 PCR on cDNA level/ all BXn and their progenies
For RNA isolation of all BXn and their progenies, material was isolated from leaves and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were amplified with anfang primers, separated on a 2% agarose gel and visualized by ethidium bromide staining.



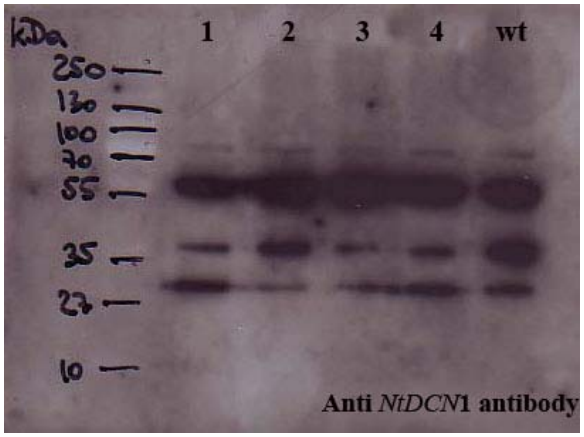
Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: wt col-O
Lane 4: 2A/2e
Lane 5: 2B/3c
Lane 6: 2D/3c
Lane 7: 2C/3d
Lane 8: 3A/2a
Lane 9: 3B/2e
Lane 10: 4A/1d
Lane 11: 4B/2d
Lane 12: 4D/2b
Lane 13: 5B/2b
Lane 14: 5C/2e
Lane 15: 5D/1d
Lane 16: 814C11/24-5-1 x wt 1a
Lane 17: 814C11/24-5-1 x wt 2a
Lane 18: 814C11/24-10-6 x wt a
Lane 19: 814C11/24- 10-10 x wt b
Lane 20: 814C11/24-5-11 x wt b
Lane 21: 814C11/24-6-10 x wt A4
Lane 22: 814C11/24-6-3 x wt B3
Lane 23: 814C11/24-6-10 x wt B4
Lane 24: 814C11/24-6-3 x wt C2
Lane 25: Negaitve Control

Fig. 57 PCR on cDNA level/ all BXn and their progenies
For RNA isolation of all BXn and their progenies, material was isolated from leaves and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were amplified with ende primers, separated on a 2% agarose gel and visualized by ethidium bromide staining

7.10. Check the efficiency of an *NtDCN*1 antibody for Western blot analysis of *Arabidopsis thaliana* backcrosses

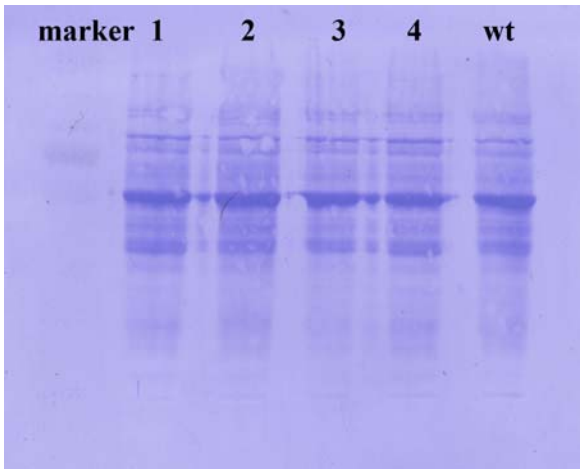
Because of the lack of an *AtDCN*1 antibody Western blot analysis was performed on *Arabidopsis thaliana*, using an *NtDCN*1 antibody. Initial Western blots on tobacco plants revealed one prominent band 30 kDa in size. If the tobacco antibody is working on Arabidopsis proteins as well we would expect one band in similar size (29k Da) on the blot.

Western blot analysis of BX1a proteins revealed four bands on the blot, one 29 kDa in size and others in sizes of approximately 37 kDa, 60 kDa and 78 kDa (Fig. 58). As a control the membrane was stained with Coomassie (Fig. 59).



Lane 1: 814C11/24-6-4_{25}
Lane 2: 814C11/24-6-4_{25}
Lane 3: 814C11/24-6-4_{23}
Lane 4: 814C11/24-6-4_{23}
Lane 5: wt col-O

Fig. 58 Western Blot
Proteins were isolated as described in Materials and Methods and blotted on a PVDF membrane for further signal detection, using an *NtDCN*1 antibody and anti-mouse alkaline phosphatase conjugate. Lanes 1-4 represent samples of BX1a, while the last lane shows the wild type control.



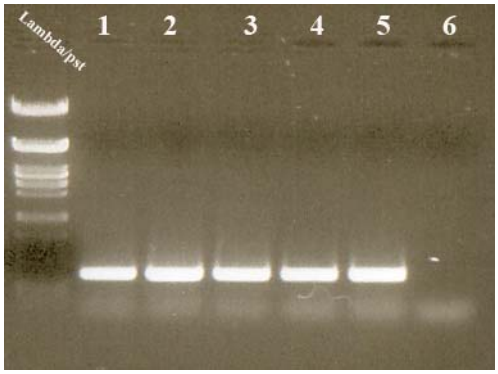
Lane 1: 814C11/24-6-4_{25}
Lane 2: 814C11/24-6-4_{25}
Lane 3: 814C11/24-6-4_{23}
Lane 4: 814C11/24-6-4_{23}
Lane 5: wt col-O

Fig. 59 Coomassie staining
To check the efficiency of protein isolation and SDS-PAGE after signal detection the membrane was stained with Coomassie

7.11. Expression pattern of *Arabidopsis thaliana DCN*1 and *DCN*1-like genes

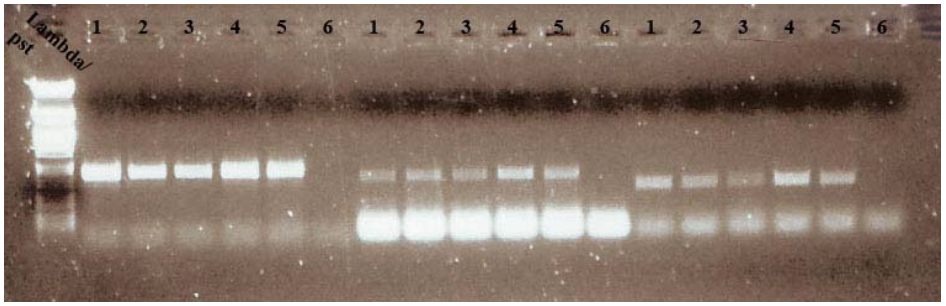
For expression patterning of *DCN*1 (At3g28970) and two *DCN*1-like genes (At3g12770, At1g15860) in *Arabidopsis thaliana* wild type plants cDNA from different organs (root, shoot, stem, flower and leave) was synthesized via RT-PCR and checked via PCR using gene specific primers for each gene, as described in Materials and Methods. As a control PCR was also performed with 18S primers (Fig. 60).

In contrast to *DCN*1-like genes very high *AtDCN*1 transcript levels could be detected in all checked organs. All three genes showed the highest expression level in leaves and flowers, while At3g12760 is strongly expressed in roots as well (Fig. 61).



1. Root
2. Stem
3. Shoot
4. Leaf
5. Flower
6. Negative Control

Fig. 60 cDNA synthesis check via PCR with 18s primers
For RNA isolation of different organs from wt col-O plants, material was isolated and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were separated on an 1% agarose gel and visualized by ethidium bromide staining.



1. Root
2. Stem
3. Shoot
4. Leaf
5. Flower
6. Negative Control

Fig. 61 Expression pattern of *AtDCN*1 and *DCN*1-like genes
RNA was isolated from from wild type col-O leaves and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were amplified with respective gene specific primers, separated on an 1% agarose gel and visualized by ethidium bromide staining. The first six lanes represent PCR products amplified with At3g12760 gene specific primers, the second six lanes show the expression pattern of At3g28970, while the last six lanes represent bands amplified with At1g15860 gene specific primers.

8. DISCUSSION

8.1. Initial research on the T2 generation of 814C11 insertion line

As the aim of this project was the molecular characterization of *AtDCN*1 (At3g12760) knock-out plants seeds of a putative *AtDCN*1 T-DNA insertion line (814C11) were ordered from GABI Kat, planted and grown to maturity for further analysis and propagation. A sequence alignment of the FST against *AtDCN*1 revealed that our gene of interest is reverse (3'-5') located within the Arabidopsis genome, while the insertion points in a 5'-3' direction (Fig. 20). GABI-Kat mentioned the insertion to be located within the 10th exon of our gene of interest (Fig. 19) and showed that the 5'- end of *AtDCN*1 overlaps with the 5'- end of the adjacent gene At3g12770 (Fig.21).

Our results obtained from PCR on genomic DNA as well as on transcript level approved the presence of a T-DNA insertion within *AtDCN*1 in three checked plants (plants 814C11/24, 814C11/25 and 814C11/27). Priming against the LB of the T-DNA and the gene revealed one band in proper size for all samples, while when using primers specific for At3g12760 on genomic DNA level no band appeared, because this part is too big to get amplified if the T-DNA insertion is located within *AtDCN*1 (Fig. 23). Because no transcript could be detected via PCR on cDNA level using At3g12760 gene specific primers as well (Fig. 24) all this results pointed to an insertion within the desired gene in the T2 generation of 814C11 T-DNA insertion plants.

To verify the presence of more insertions within the mutant genome, which could disrupt other genes in addition to *AtDCN*1 Southern blot analysis was performed. Using a LB probe for hybridization, two up to four insertions for different T2 plants using restriction endonuclease *EcoRI* were revealed, while when performing double digestion (*PvuII/ HindIII*) two up to three signals could be detected (Fig. 22). Comparing both restriction digests on the blot plants 814C11/24 and 814C11/25 showed the fewest signals and were therefore candidates for further backcrossing to a wild type background to cross out the additional determined insertion(s).

8.2. Backcrossing of multiple insertion *AtDCN*1 T-DNA mutant plants with a wild type col-O background

To segregate out the excessive insertion(s) within the 814C11 genome we had to backcross mutant plants with a wild type col-O background, as described in Materials and Methods.

Two independent backcross approaches (BX1, BX2) were performed with plants of the T2 generation of 814C11. In BX1 we concentrated our work on emasculating 814C11/24 plants to pollinate them with wild type pollen, while in BX2 we tried different backcross variations. To ensure a successful result, another backcross (BX3) was performed with plants grown from BX1 seeds and a wild type col-O background (Fig.62).

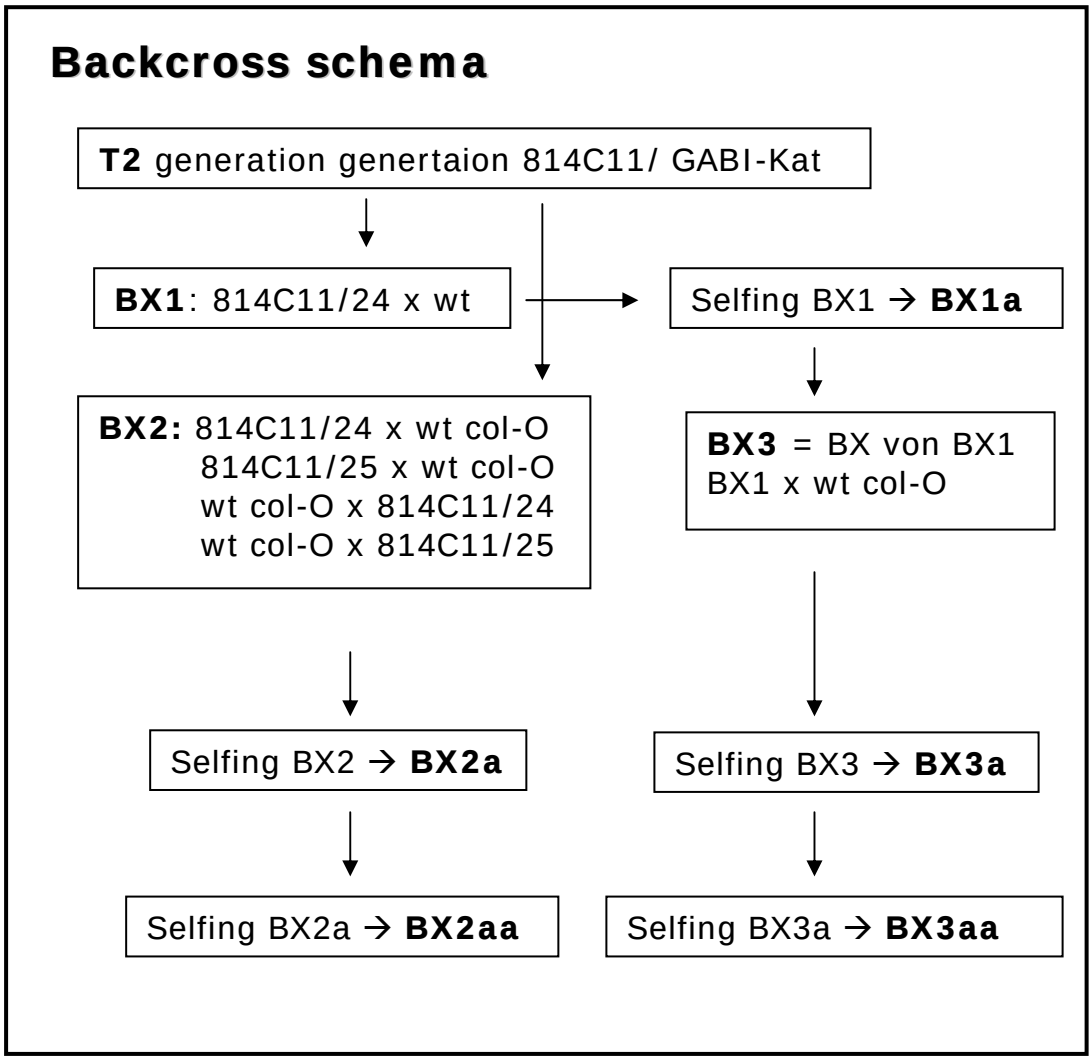


Fig. 62 Backcross schema

This Figure gives an overview about the different backcrosses, which were done during this project. Backcrosses are designated BX, their progenies BXa , while progenies of Bxa are designated as BXaa, and so on. In this Fig. plant IDs are separated with an x, which means that plant one (emasculated, female part) was crossed (x) with a second plant (male part).

8.3. Genotyping of backcrosses and their progenies

As the backcrossed siliques were mature, they were collected and planted for checking the situation after backcrossing via PCR on genomic DNA level. All BX1a plants showed the presence of a T-DNA insertion within the desired gene, using an *AtDCN*1 gene specific primer and a T-DNA LB one (Fig. 25, Fig. 26, Tab.1). As a second proof, primers At3g12760 for and rev were used for genotyping on genomic DNA level as well. If the T-DNA insertion is within *AtDCN*1, this part would be too big to get amplified with the same PCR program, hence expecting no signal for successfully backcrossed plants (Fig. 27, Fig. 28, Tab. 2). Three BX1a samples (814C11/24-6-4, 814C11/24-6-7, 814C11/24-6-9) showed the desired result and were considered for further analysis and propagation.

BX2a and BX3a plants were genotyped on genomic DNA level as well, using an *AtDCN*1 gene specific primer in combination with either a T-DNA LB or RB primer. This large scale scanning proved by both primer pairs revealed a few positive plants of BX2a (2B/2b, 2C/1a, 2D/2b, 3B/2b, 4A/1c, 5B/1a, 5C/2d) and one of BX3a, namely 814C11/24-6-10 x wt A2 (Fig. 29- Fig. 32, Tab. 3).

8.4. Expression analysis of backcrosses and their progenies

To confirm data revealed from experiments on genomic DNA we had to proof the results on transcript level. A total of five plants showed positive results on gDNA level and lack a proper *AtDCN*1 transcript, namely BX1 progenies 814C11/24-6-4_{23} and 814C11/24-6-4_{25}, BX2 progenies 2B/3c and 4B/2d, and one progeny of BX3 814C11/24-6-3 x wt A4 (Fig. 33, Fig. 34, Tab.4). Data obtained from genotyping and expression analysis point to an insertion within *AtDCN*1 for above mentioned plants, but do not predicate anything about how many insertions are located within the 814C11 mutant genome.

GABI-Kat mentioned a possible second insertion to be located in At2g28540 or in At3g12770, respectively which was also checked on transcript level. Weak to strong signals for transcripts of both genes could be detected via PCR using primers specific for each gene, while checking BX1a as well as BX2a plants (Fig. 35, Fig. 36). Those results could be interpreted in three ways: First, the backcrosses worked properly and the second insertion which was also detected via Southern blot analysis of T2 plants was segregated out; another

option would be that the second insertion is located in any other gene than mentioned by GABI-Kat; third, both insertions are located adjacent within *AtDCN*1 hence no recombination event can occur. If the last situation is the case it would be impossible to cross out the second insertion via backcrossing.

8.5. Southern blot analysis of different backcrosses and their progenies

To find any evidence for one of the above mentioned hypothesis large scale scanning via Southern blot analysis of all backcrosses and their progenies was performed with new probes designed to bind to the LB, or to the middle of the T-DNA (RB), respectively (Fig. 63).

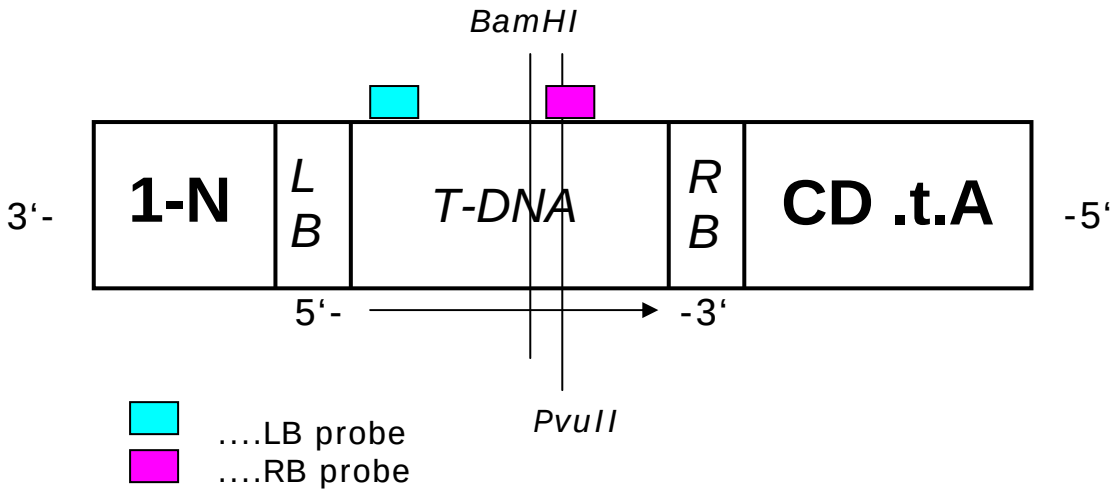


Fig. 63 Schema of Southern probes RB and LB binding sites

After digesting genomic DNA (*BamHI BamHI/ PvuII*) the fragments were separated on an 0,8% agarose gel, blotted on a Nylon membrane and hybridized with the first probe (RB). The restriction endonuclease *BamHI* cuts once in front of the RB probe binding site; not elsewhere in the insertion but for sure somewhere in the plant's genome. If the first mentioned hypothesis namely the out-segregation of one of the initially determined insertions is our case, we would expect one band with unknown size on the blot. *PvuII* cuts once within the RB probe binding site hence performing a *BamHI/ PvuII* double restriction, we would expect two smaller bands on the blot whose sum is the size of the big band appearing, when cut with *BamHI.*

To confirm blotting data the membranes were stripped with 0,1% SDS and hybridized with a second probe (second LB probe). The results when cut with *BamHI* were expected to

be similar to the previous; one band of unknown size on the blot if the backcross was successful. Performing *BamHI/ PvuII* double digestion would not change the results awaited from *BamHI* single digestion because the second enzyme cuts outside of the LB probe binding site.

If one of the other hypothesis is our current status of research we would expect more signals on the blot either using the RB or LB probe.

Southern blot analysis of BX2a plants confirmed the first hypothesis; one signal, 6,5 kb in size was detected when digested with *BamHI* and probed against [α-^{32}P]dCTP-labelled RB fragments. Using the same probe and genotypes of BX2a plants but two restriction enzymes (*BamHI/PvuII*) the expected two signals were detected on the blot. The appearance of a 6,5 kb signal occurred because of some left uncut DNA; apparent *PvuII* did not cut the whole applied amount of DNA properly resulting in one band similar to obtained results from single digestion (6.5 kb), and in two smaller bands (2 kb and 4.6 kb) (Fig. 41).

Previous Southern blot data pointed to one insertion within the backcrossed plants which was proven via re-hybridization of the membrane with [α-^{32}P]dCTP-labelled LB fragments. Digesting BX2a samples with *BamHI* revealed 4,5 kb and 8-9 kb signals for lanes 1a-3a, 5a and 6a; lane 4a also showed two signals but in different sizes (2,8 kb and 7kb), while lane 7a showed four bands (2,8 kb, 4,5 kb, 7 kb and 8-9 kb) on the blot. Performing a *BamHI/ PvuII*, double digestion three more signals occurred in addition to some bands appearing when cut with *BamHI*. Lanes 1b-3b and 5b showed two bands 4 kb and 4,5 kb in size; lane 4b showed 5 signals (2,2 kb, 2,8 kb, 4 kb, 4,5 kb and 7 kb), while lanes 6b and 7b showed three signals, 2,2 kb, 2,8 kb and 7 kb in size (Fig. 42). Those unexpected results adduced the conclusion that there have to be more insertions within the mutant genome or within *AtDCN*1, respectively.

Similar results were obtained checking other BX2a plants and one sample of BX3a (Fig. 44, Fig. 45).

Divergent results were revealed checking some more BX2a plants as well as BX3 and their progenies. Most of them did not show a proper signal probing against the RB. For some BX3a samples (814C11/24-6-10 x wt B2, 814C11/24-6-3 x wt C1,814C11/24-6-10 x wt A1), one strong specific band 6,5 kb in size occurred, while for three BX2a samples (2B/2c, 4A/2b 5B/1c) just a very weak signal in the same size could be detected (Fig. 47, Fig. 49).

8.6. Check for multiple insertions within *AtDCN*1

Results obtained from Southern blot analysis point to more insertions located within the mutant's genome or within *AtDCN*1, respectively. To verify this, every possible situation was considered and checked via expecting and estimating the sizes of signals obtained via DNA blotting, using different restriction enzymes and probes.

Just one hit showed every expected band on seven different blots, namely the situation when two insertions are located RB to RB within *AtDCN*1 (Tab. 5, Fig. 11).

Situation: 2 T-DNA insertion RB to RB within At3g12760

Restriction enzymes	expected sizes of signals	Size of signal on the blot	Southern Blot probe
HindIII/PvuI	2.3 kb + 3.4 kb	2.3 + 3.2 (weak)	First LB
EcoRI	2.7 kb + 1.9 kb	2.7 + 1.9 kb	First LB
BamHI	4.5 kb + ? kb (large signal)	4.5 kb + 8-9 kb	second LB
BamHI	6.6 kb	6.5 kb	RB

Tab. 5 Overview Southern blot results

To further prove the predicted situation PCR on genomic DNA was performed with primers that amplify 400 or 900 bp around adjacent RBs, respectively. Results from PCR with both primer pairs also point to the situation of two insertions located inverse within *AtDCN*1, although one checked plant (BX2a: 4A/1c) showed a second signal after amplification with primers RB 400 (Fig. 50, Fig. 51).

8.7. Expression analysis via Northern blotting of different backcrosses and their progenies

Northern blot analysis was performed to approve data obtained from expression analysis, namely that no *AtDCN*1 transcript is present in backcrossed plants. Results obtained from RNA blot analysis revealed weak up to strong signals for all BX2a plants (Fig. 52, Fig. 55), as

well as BX3 and BX3a samples (Fig. 53, Fig. 55) which came as a big surprise and awaited further interpretation.

As mentioned in the introduction the *Arabidopsis thaliana* genome contains one *DCN*1 homolog and two *DCN*1-like genes with high sequence similarity and identity. The probe used for Northern blotting (wild type cDNA amplified with At3g12760 gene specific primers) could anyhow hybridize to At3g28970 and At1g15860 *DCN*1-like genes, though with less specificity than to the *Arabidopsis thaliana DCN*1 homolog At3g12760. Although At3g12760 is knocked-out by two T-DNA insertions the probe would detect the intact *DCN*1-like genes revealing a weaker signal on the blot. Comparing Northern blot results (Fig. 52, Fig. 53, Fig. 55) with PCR data obtained from amplification of gDNA as well as cDNA (Tab.3, Tab. 4), one BX2a plant (4B/3e) and one BX3a plant (814C11/24-6-10 x wt A5) would fit to this hypothesis. Both backcrossed plants were shown to have an insertion within At3g12760, lack the proper transcript and show a weak signal detected via Northern blot analysis.

Another possible interpretation would be following: The T-DNA insertion is located in the 10^{th} exon of At3g12760, hence at the very 5'- end of the gene (Fig. 19). The plant's polymerase may transcribe the gene up to the beginning of the insertion, then falling off resulting in expression of a truncated version of the transcript. If true, it would be obvious that no *AtDCN*1 transcript could be detected via expression analysis on cDNA level using At3g12760 gene specific primers which anneal to the 3'- or 5'- end of the gene, respectively (Tab. 4) and would explain the appearance of proper transcripts via Northern blot analysis (Fig. 52, Fig. 53, Fig. 55). Unfortunately, this hypothesis was rather dismissed because almost all plants showed the presence of either the 3'- or the 5'- end of an *AtDCN*1 transcript, while just a few were shown to lack this parts. (Fig. 50, Fig. 51, Tab. 4). On the other hand results obtained from this PCR approach functioned as an additional proof for the overall expression of *AtDCN*1; because plants which were shown to miss a complete *AtDCN*1 transcript should obviously lack the 3'- and 5'end of the transcript too. After comparing all data obtained from genotyping and expression analysis of different backcrosses and their progenies just one BX3a sample (814C11/24-6-10 x wt A5) among plants which were shown to lack the 3'- or 5'- end of *AtDCN*1, could be identified as positive mutant plant. The backcross 814C11/24-6-10 x wt A5 was also shown to have a T-DNA insertion within *AtDCN*1 examined on gDNA level using two different primer pairs and was shown to miss an overall transcript checked on cDNA level (Tab. 3 ,Tab. 4, Fig. 65).

8.8. Western blot analysis of *Arabidopsis thaliana* with an *NtDCN*1 antibody

Because of the lack of a specific *AtDCN*1 antibody the efficiency of an *NtDCN*1 antibody was checked on *Arabidopsis thaliana* protein extracts. Initial research on tobacco plants using an *NtDCN*1 antibody revealed one specific band, 30 kDa in size (data not shown). *AtDCN*1 encodes a 29 kDa protein hence we would expect one band, 29 kDa in size if the tobacco antibody is working for Arabidopsis as well.

The appearance of four bands (29 kDa , 37 kDa, 60 kDa and 78 kDa), lead to the conclusion that the tobacco antibody is too unspecific for analysis on *Arabidopsis thaliana* proteins (Fig. 58). Furthermore no significant differences between wild type col-O and probably positive *AtDCN*1 mutants could be detected, except a stronger 37 kDA signal appearing in the wild type control.

8.9. Phenotypes, germination rates and segregation analysis

Because our previous research on tobacco clearly indicated that *NtDCN*1 is involved in phase changes during gametophyte development and embryogenesis in plants, we awaited an obvious phenotype for *Arabidopsis thaliana* backcrosses as well.

BX1, BX2 and their progenies had smaller rosette leaves and showed a lower overall height than wild type plants when grown under green house conditions (Fig. 64). On the other hand almost all BX3 plants and their progenies were taller than wild type plants and had bigger rosette leaves. By checking the germination rates of almost every backcross we could not observe any abnormality divergent to wild type rates, aside some BX1a plants which show a lower germination rate than wild type seeds (Tab. 6- Tab.9). Also DAPI staining of interesting backcrosses and wild type pollen did not reveal any abnormalities in the nuclear organisation or structure of the pollen (data not shown).

Fig. 64 Phenotypic analysis
This picture shows a wild type col-O (left side) and a representative of BX2 (right side), three weeks after planting seedling into soil..

Before further transformation of homozygous *AtDCN*1 plants with two T-DNA insertions within the desired gene with constructs mentioned in consecutive project ideas, segregation analysis has to be performed. Sterilized seeds of BX1 plants (BX1a seeds) and wild type col-O controls were spread out on MS medium containing either Kanamycin or Sulfadiazin; as a control the germination rate was also checked on MS medium without addition of any antibiotics. Both wild type controls nearly showed germination rates up to 100% on MS medium without any antibiotics while BX1a rates varied between 41,2% to 88,23% (Tab. 6).

Medium	Genotype	% germination
w/o	wt col-O a	100
w/o	wt col-O b	94,4
w/o	BX1a/814C11/24-6-4/23 a	66,6
w/o	BX1a/814C11/24-6-4/23 b	81,25
w/o	BX1a/814C11/24-6-4/25 a	88,23
w/o	BX1a/814C11/24-6-4/25 b	41,18
Kanamycin	wt col-O a	0
Kanamycin	wt col-O b	0
Kanamycin	BX1a/814C11/24-6-4/23 a	0
Kanamycin	BX1a/814C11/24-6-4/25 a	0
Sulfadiazin	wt col-O a	0
Sulfadiazin	BX1a/814C11/24-6-4/23 a	61
Sulfadiazin	BX1a/814C11/24-6-4/25 a	94,4

Tab. 6 Segregation analysis and germination rates of BX1a

Backcrosses contain a Sulfadiazin resistance in their genome because of former transformation with pAC106, a Sulfadiazin resistance containing vector (Fig. 18), hence all plants which have at least one T-DNA insertion within their genome should grow on this antibiotic, in contrast to wild type seeds. While all wild type plants bleached out and died within ten days BX1a seeds showed germination rates of 61% to 94,4% (Tab. 6). For further transformation of *AtDCN*1 backcrosses with different constructs a vector containing another resistance gene than Sulfadiazin has to be used for selection of positive transformants. All seedlings bleached out and died within ten days when spread out on MS medium containing Kanamycin hence a vector containing this resistance gene could be used for further transformation and selection (Tab. 6).

BX2a germination rates		
	plant A emasculated x plant B	**germination %**
plate #1 A/1	wt col-0	100%
plate #1 B/1	wt col-0 4	87,50%
plate #2 A/1	814C11/24-3-1 x wt col-0 4-4	100%
plate #2 A/2	814C11/24-3-1 x wt col-0 4-4	100%
plate #2 A/3	814C11/24-3-1 x wt col-0 4-4	83,30%
plate #2 B/1	814C11/24-4-2 x wt col-0 7-5	86%
plate #2 B/2	814C11/24-4-2 x wt col-0 7-5	88%
plate #2 B/3	814C11/24-4-2 x wt col-0 7-5	100%
plate #2 C/1	814C11/24-5-1 x wt col-0 7-4	85%
plate #2 C/3	814C11/24-5-1 x wt col-0 7-4	96%
plate #2 D/2	814C11/24-2-4 x wt col-0 2-1	100%
plate #2 D/3	814C11/24-2-4 x wt col-0 2-1	79%
plate #3 A/1	814C11/25-2-2 x wt col-0 4-3	100%
plate #3 A/2	814C11/25-2-2 x wt col-0 4-3	100%
plate #3 B/1	814C11/25-3-5 x wt col-0 2-2	94%
plate #3 B/2	814C11/25-3-5 x wt col-0 2-2	100%
plate #3 B/3	814C11/25-3-5 x wt col-0 2-2	100%
plate #3 D/1	814C11/25-4-5 x wt col-0 2-1	94%
plate #3 D/2	814C11/25-4-5 x wt col-0 2-1	95%
plate #3 D/3	814C11/25-4-5 x wt col-0 2-1	96%
Plate #4 A/1	wt col-0 6-5 x 814C11/24-7-4	100%
Plate #4 A/2	wt col-0 6-5 x 814C11/24-7-4	93%
Plate #4 A/3	wt col-0 6-5 x 814C11/24-7-4	100%
Plate #4 B/2	wt col-0 1-3 x 814C11/24-2-5	100%
Plate #4 B/3	wt col-0 1-3 x 814C11/24-2-5	100%
Plate #4 D/1	wt col-0 5-5 x 814C11/24-4-1	100%

Plate #5 B/1	wt col-0 5-1 x 814C11/25-5-4	100%
Plate #5 B/2	wt col-0 5-1 x 814C11/25-5-4	100%
Plate #5 C/1	wt col-0 6-4 x 814C11/25-4-1	85%
Plate #5 C/2	wt col-0 6-4 x 814C11/25-4-1	100%
Plate #5 C/3	wt col-0 6-4 x 814C11/25-4-1	100%
Plate #5 D/1	wt col-0 1-2 x 814C11/25-4-1	100%
Plate #5 D/2	wt col-0 1-2 x 814C11/25-4-1	100%

Tab. 7 BX2 plant IDs and plant germination rates

BX3/germination rates		
plant A emasculated	**plant B**	**Germination %**
814C11/24-5-1/1	wt e	100%
814C11/24-5-1/2	wt e	100%
814C11/24-5-1/3	wt e	100%
814C11/24-10-10	wt j	88,80%
814C11/24-5-11	wt h	100%
814C11/24-10-6	wt e	66,60%
814C11/24-5-5	wt e	100%
Wt col-O j		98,50%

Tab. 8 Germination rates of BX3 seeds

BX3a/germination rates		
plant A emasculated	**plant B**	**Germination %**
814C11/24-6-3 B	wt j	100%
814C11/24-6-3 C	wt j	100%
814C11/24-6-10 A	wt h	100%
814C11/24-6-10 B	wt h	100%

Tab. 9 Germination rates of BX3a seeds

8.10. Expression pattern of *AtDCN*1-like genes in different organs

Because one *DCN*1 homolog (At3g12760) and two *DCN*1-like genes (At3g28970, At1g15860) are existing in the *Arabidopsis thaliana* genome we decided to check their expression pattern in different organs of wild type plants via PCR using respective gene specific primers.

At3g12760 (*AtDCN*1) showed the most intensive signals in all check organs, indicating the highest expression level in comparison to *DCN*1-like genes. At3g12760, At3g28970 and At1g15860 transcripts showed the highest expression in leaves and flowers, while At3g12760 was strongly expressed in roots as well (Fig. 61).

9. SHORT RESUME

Because all results obtained during this diploma thesis except expression analysis via Northern blotting are congruent, the current state of research is following: Two T-DNA insertions are located inverse (RB to RB) within *AtDCN*1 hence the out segregation of the second insertion is not possible, but also not required for further analysis of backcrossed plants. PCR on genomic and on cDNA level as well as Southern blot analysis lead to the identification of one positive BX3a plant (814C11/24-6-10 x wt A5) which is homozygous for the mutation. The unexplainable results obtained from Northern blot analysis and the lack of an obvious phenotype could be due to sequence similarity or redundancy of one *Arabidopsis thaliana DCN*1 homolog and two *DCN*1-like genes, respectively.

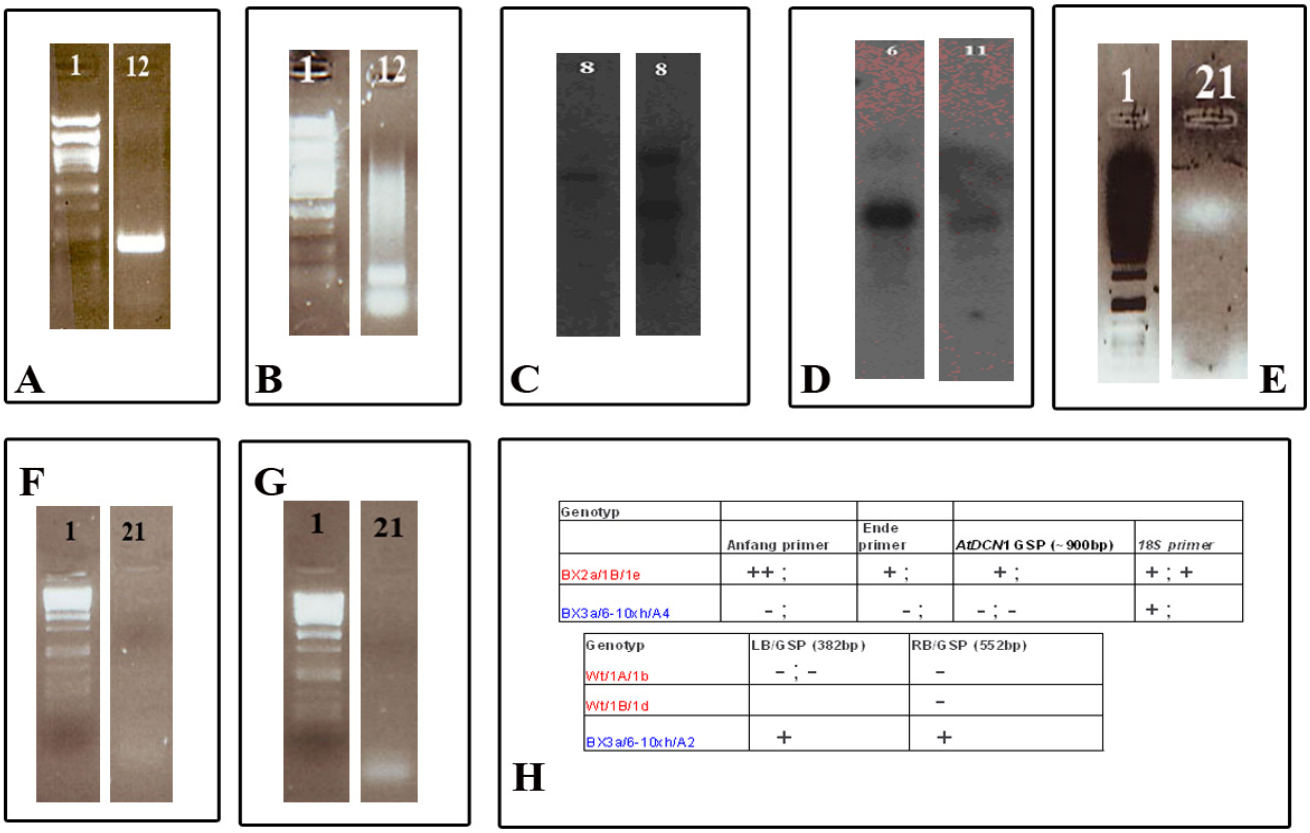


Fig. 65 Overview of results from the positive BX3a mutant 814C11/24-6-10 x wt A5
A: Results from PCR on gDNA level with primers T-DNA LB/ 2 and GSP, lane 1 represents a Lambda/ pst marker while lane 12 shows 814C11/24-6-10 x wt A5; B: Results from PCR on gDNA level with primers GSP/ 2 and RB, lane 1 represents a Lambda/ pst marker while lane 12 shows 814C11/24-6-10 x wt A5; C: Results from Southern blot analysis of 814C11/24-6-10 x wt A5 hybridized with RB (left picture) and LB (right picture) probe, D: Results from Northern blot analysis, lane 6 represents a wild type control, while lane 11 shows 814C11/24-6-10 x wt A5; E: Results from expression analysis on cDNA level with At3g12760 gene specific primers, lane 1 represents a Lambda/ pst marker while lane 21 shows 814C11/24-6-10 x wt A5, F: Results from PCR on cDNA level with primers anfang, lane 1 represents a Lambda/ pst marker while lane 21 shows 814C11/24-6-10 x wt A5; G: Results from PCR on cDNA level with primers ende, lane 1 represents a Lambda/ pst marker while lane 21 shows 814C11/24-6-10 x wt A5.

10. CONSECUTIVE PROJECT IDEAS

For further functional analysis of *AtDCN*1 the identified positive mutant plant BX3a 814C11/24-6-10 x wt A5 could be transformed with following constructs:

- Overexpression construct with 35S, DC3 and/or inducible promoters
- Promoter GUS construct with own (At3g12760) promoter
- Complementation
    - cDNA construct with 35S promoter
    - cDNA construct with own promoter
    - gDNA construct with own and/or 35S promoter

Additionally analysis on protein level could help to characterize the biological and biochemical function of *AtDCN*1 and *AtDCN*1-like genes; hence antibodies against DCN1 and the two DCN1-like proteins would have to be generated.

11. LIST of ABBREVIATIONS

~…….approximately
°C ……. degree
µg ……..micro gram
µL …….micro litre
µM…….micro molar
bp …….… base pairs
BX ……..…backcross
Bxa ……progeny of backcross
cDNA……complementary DNA
cm……..centimetre
cps……..counts per minute
Fig……..figure
FST……flanking sequence tag
g ……gram
gDNA……genomic DNA
GSP……gene specific primer
h…….hour
hrs……..hours
kb……...kilo bases
L ……litre
LB……..left boarder
M……Molar
mg …….milligram
mL …….millilitre
mM……milli molar
mm…….millimetre
$N_{2\ liquid}$ ……...liquid nitrogen
nm .....…nanometre
o/n……..over night
PCR……polymerase chain reaction
RB……..right boarder
RNAi……..RNA interference
rpm……rotations per minute
SDS-PAGE……sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tab…….table
UV ……ultra violett
V ……volt
w/o…….with out
wt col-O ……wild type Columbia O

β-ME ……beta mercaptoethanol
APS……ammoniumperoxodisulfat
BSA……...bovine serum albumin
DAPI……4',6-diamidino-2-phenylindole
ddH_2O^a ……..double distilled, autoclaved water
DEPC……diethylpyrocarbonate
dH_2O……..distilled water
DTT……. dithiothreitol
EDTA……ethylenediaminetetraacetic acid
EtBr ……...ethidium bromide
EtOH ……ethanol
HCl……hydrochloric acid
KCl……... potassium chloride
KH_2PO_4…….monopotassium phosphate
$MgCl_2$……....magnesium chloride
MOPS.....3-(N-morpholino)propanesulfonic acid
MS ……Murashige and Skoog

Na acetate……..sodium acetat
Na……..sodium
Na_2HPO_4……...disodium hydrogen phosphate
NaCl……...sodium chloride
NaH_2PO_4……...monosodium phosphate
NaOH……sodium hydroxide
$NaPO_4$……sodium phosphate
PMSF……phenylmethanesulphonylfluoride
SSC……saline-sodium citrate
TEMED…… tetramethylethylenediamine
Tris……trisamine

12. LIST of FIGURES and TABLES

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14. SEQUENCES

14.1. At3g12760 genomic DNA sequence

ATTTTGCATAGAAGAGAGAGAGGCGGAGAGAGAGGAAAGTGAAGAAGCAATCGTGAGAGACTCT
CTCGAGTCTTCCCTGAGTCACTCCTCTCCTCTCACACCTTTTTGCTTCGCGATTAAGAGTCAGAGAG
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TCTTCTCATTATTTTAAATAGACTTGCTTATCTCACAGATTTTTCTATTGTGTTTGTCAGAATCTTTA
AATTGATGATTAAATAAGTAAGCTTTTCAACCTTAAGACT

14.2. At3g12760 CDS

ATGCATAAGTTGAGCAGAAGCAACCGTGACAAACTTCAGCAGTTCGTGGCTATCACAGGAGCTAGT
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GCAGTTGCTCTTTGCAGAAAGAGAGTGGCCTTTGGTAACTCACTGGTGTGATTTCTTGCAGGATCGT
CATAACAAGGCCATATCTAAGGACACATGGGCACAGCTACTGGAATTTTCAAGGATGGTCGACCCA
GTGCTGTCGAATTACGATGCAGAAGGAGCATGGCCTTACCTGATTGATGAATTCGTTGAGTATTTG
TATGACAAAAATGTCGTTGAGAAGTGA

14.3. At3g28970/AAR3 CDS

>gi|28973570|gb|BT005690.1| Arabidopsis thaliana clone U51023 unknown protein

(At3g28970) mRNA, complete cds/AAR3

ATGGATTCTTCGCCAGTTTCTGCTCGATTCGATATATTCGAGATTTATCGTAGATTCTGTGCTATTCG
ATCAGGACAACAATTGTGTAATAAGAAACCCTGTGATGGCGAAGAATCTCAAAGATTTAACTTGTC
TAAAGAAGCAATAACTCAGCTCTTATATCTAGTAGAGAATAAGTTTCAAGCTAGGAACTCGATTTT
TGATGAATTGTTTAAGTTGATGTCACGGCTAGACTTAATGGTGGACTTTACGGAGTTTACTTGCTTT
TACGATTTTGTTTTCTTTATGTGCCGCGAGAATGGGCAAAAGAATATAACTATAAGTAGAGCTATT
ACTGCATGGAAACTTGTTTTGGCTGGGAGATTTAGGTTACTTAACCGCTGGTGTGACTTTATCGAGA
AAAATCAACGGCACAATATTTCAGAAGATACTTGGCAACAAGTCTTGGCGTTTAGTCGTTGTGTAC
ATGAGAATCTAGAAGGATATGACTCTGAAGGTGCATGGCCTGTCCTAATAGATGACTTTGTCGAGC
ATATGTACAGTATTTTAGGACCGAATAAGGACACTAGCTTGTTCTGCAAATGTGGTGACACAGAAT
CTGAATCATGCCTCTATCAAGAAGATGAGCATCACAAAGATTATCGCCGCCCACACACGGGTTTAA
GAAACATTCCAGGTTTAAAGAGAAAAACATCGAAGAAGAATGACGAAGAAGAAGAAGATGAAGA
TGAAGAAGTGTTAGAAACCCAAAACTCTTCTTCTTTGCTAAACTTCAAACGAATCAAAACCAGCAA
CAGTCCAAGGTGTTCATCAAAGTCCCCTTGTTCAATCGAGCGAAGTCTTTCACAAGGTTTTGCGAGT
CTTTTATCGACTGGAGATAAACCATGAAACACTGGAACATCGTCGCACATATATATAG

14.4. At1g15860 CDS

>gi|32815874|gb|BT009700.1| Arabidopsis thaliana At1g15860 gene, complete cds

ATGCGTCGCTCTTCATCAAAGAAGAAATCAGGTCAATCAACTGAATCAGTCACCACGGATCTCTTT
CGCTCAGCTTCGAGCAAGGCCTCGAATAAAGAGATGGATCGAATTGATCACTTATTTAATCAGTAT
GCCAATAAATCTTCCAGCCTGATTGATCCTGAAGGAATAGAGGAACTATGCTCCAATTTGGAAGTG
TCACATACTGATATCAGAATCTTGATGCTTGCTTGGAAGATGAAAGCTGAGAAACAAGGTTACTTT
ACACATGAGGAGTGGAGAAGAGGCCTCAAGGCTTTAAGAGCTGATACGATCAATAAGTTGAAGAA
AGCCCTTCCGGAGCTTGAGAAAGAGGTCAGGAGGCCATCAAATTTTGCAGATTTCTATGCTTATGC
CTTCTGTTATTGTTTAACAGAGGAAAAACAGAAGAGCATAGACATAGAGACTATATGTCAACTCCT
AGAGATCGTCATGGGATCTACATTTCGAGCCCAAGTTGACTACTTTGTTGAGTATTTAAAGATCCA
AAACGACTACAAAGTGATAAACATGGACCAATGGATGGGACTTTACCGGTTCTGTAACGAGATAA
GTTTCCCGGACATGGGGGACTATAATCCAGAGCTTGCATGGCCATTGATTCTTGACAATTTTGTTGA
GTGGATTCAAGAAAAACAAGCCTGA

14.5. At1g28540 full length cDNA

GAACAAAAGGTTTAGCCACAGTTTTTTTTTTTCTCCTCGTCTCTTCGCAAATCCTCTTCTTCCTTCGA
TTCTCTCTGAAAATCTCTTCTCCTTCCTCAAAAACCGATACTCTCTTCTTCAATCTCCCTCTCTTATCT
CTCCAAGCTAATTCTCCAAACCGTCTTTTCTCAAACCCTAACGTACGGATTAGGGTTTTTGTTCGTT
GATTCCAAAGGTTACGACTTTCAATTTCTCCACCAAATTCTCTGTAAAGGTGACGATGAATGAGAA
AGGAGAAAAGACTTGTCCTCTATGTACTGAGGAGATGGACTTGACTGATCAACATCTTAAACCTTG
CAAATGTGGTTATCAGATATGTGTTTGGTGTTGGCATCATATTATAGAGATGGCTGAGAAAGATAA
GACAGAGGGTCGTTGTCCTGCATGTCGTACCCGTTATGATAAGGAAAAGATTGTAGGGATGACAGT
CAGCTGTGAAAGGTTGGTTGCTGAATTTTACATTGACCGGAAAAAGTCACAGAAGGCGAAGCCTA
AACCTGCGGAGGGAAGGAAAGATCTAACTGGTGTGAGAGTTATTCAGAGAAACTTGGTTTACGTTA
TGAGCTTGCCTTTTGACCTGGCAGATGAAGATATGTTTCAGCGAAGAGAATACTTTGGTCAATATG
GGAAAGTCGTCAAAGTAGCAATGTCTCGGACTGCAGCTGGGGCTGTCCAACAATTTCCAAACAATA
CTTGCAGTGTATATATTACTTACTCCAAAGAGGAGGAAGCGATTCGATGTATCCGATCAGTACATG
GATTTATATTGGATGGTAGAAATTTGAAGGCATGTTTTGGAACCATGAAATATTGTCATGCGTGGTT
GAGAAACATGCCTTGCTCCAATGCTGAATGCCTTTATTTGCATGAGATTGGTGCCCAAGAGGATAG
TTTCTCAAAAGATGAGACCATATCAGCGCACATGAGGAAAATGGTTCAAGATATTACTGGTTGGAG
GGATCATTATGTGCGGCGTTCAGGGAGCATGTTACCTCCACCAGTTGATGATTATGTTGATAATGA
GTCTAGCACAAGAATTATTCCAAAAGTTGTTTTGAATAATGTACATAGTGCCGCGAAAAATTCTCC
TCCAAATGACAGCAACAGTCACTCTGTTACTCTTCCTGCTGGAGCCATGTGGGGAATGCATTCTTCG
GTACCGAATACACCGTCTTCTAGGGAACCCTTAAGAGACAAATCTGCTACAGTTTCATCTGCGGTT
GCAATCAATCCTACACAGATCTCTTCTCGTAGTGATGAATTAAGGAAGCCAGCATTAGAGGCTGCA
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GCCCAATCAGTTAAACGGCAGTCTTAACCATTCTATTTTGGTTCCAGACAAAGCAAGGGACACACA
GCCAATTGAAAATTGTTTCGTTGACTCCCATGAAAGCCCAAGTGAAATAGATGACAGGATAATTGC
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GAAAGTGACGAAGAGGCCAGGTCTCTTAAGCTAGCCAGTTCCAGTAAAGTTGAGGATAATCAATC
CCGGTTTTCTTTTGCCAGGCAAGAGGAACCAAAGGATCAAGCTTTTGATTCTTACAACGCCTCTAAC
CAGATGTCACGTGGCAATGACTTCTACCAGAATTCTTCAGAACGACAGAGTCCTAATATGGGTATG
TTTGGGACCTATAATGGTCTTTCATCTTGTTATCGCAGGGGACTAGACTACGTCACGGAAAGCTCCA
CTTTGCCCTCATCCTATAAACCTACTTCTGTTCCAAGATGTCCGGTTTCAGCACCACCTGGCTTTTCA

GTTCCAACTCCAAGTCGACCTCCTCCTCCAGGCTTCTCCTCAAATGGGAGAGACCATCAGATGATT
GATGGCTTTTCAGGAAACTCTCGTTTTTCTGATTCAATTGCGTACGGTAATCACTATCAGCAGTCAC
TTCCGATTGAAAATGTGAGGGATGTTCAATACATGGATCCTGCCATTTTGGCCGTTGGTCAAGGCTT
TGAGAATGCAAGCCTAGATTTCAGATCAAACTTTCAAGGAAACACAAACATGTATGGAAGCGCGG
CAAAGCTCCAACAACAACAACAACAAGCGGTAATGCAGAACCCTTTGTCTATGCAGAACCCTTTGT
CTTCGCATCAAAACTGTAGATTCACTGATTCTTTGGGAATGGCACCAAGGTTTATAGATCAATCTCA
AGGCAATAACTTACTGACTAGAAATATGGCCTTGCCTAATGGTCACTGGAATGGGTTGATGAGTAA
CGAGATCCAAACTCGAAACAGACTTCAAAATGAGAGACTTGTTGGTTCGACTAATTGGATCCCTGG
TTACAATGGGACGTTCAGGATGTAAACACCGTCTGCGGAATCTGTGAGCTTGGTTTGCCTTTGGACT
GACTGATAGGAACCATCATCGGTTTGGTCTAGGAGCCGTGCTTAAATCACCTCGGCCTTAAATCTTT
TCTTCTGGTTACTTAGTAGTAAAGTTGGTGTAAAAAAAGCTTTAGGAAGGTTTTTTATTCGTTTATG
CAATTCTATAACATTTACTCTTGGTTTTGAAGTATGTCAAATATGGATTTTCGAGGTT

14.6. At3g12770 full length cDNA

AAGGAAGACAGAGTAATAACCATGTCGGAAGCATCTTGTCTTGCTTCTCCTTTGCTCTACACCAATT
CTGGAATCCATTCTGATTCTTTCTACGCTTCGCTTATTGATAGTGCCACTCATAAAGCTCAGCTGAA
GCAAATCCACGCACGTTTACTTGTTTTAGGTTTGCAGTTCAGTGGTTTCTTGATCACCAAGCTCATT
CACGCTAGCTCTTCCTTTGGTGACATTACTTTTGCACGCCAAGTGTTCGACGATTTACCTCGTCCTC
AAATATTCCCTTGGAATGCTATAATCAGGGGTTATTCAAGGAACAATCACTTCCAAGATGCTCTTCT
CATGTATTCCAATATGCAACTCGCTCGTGTTTCTCCTGATTCTTTCACTTTCCCTCATCTTCTTAAAG
CTTGCAGCGGTTTGTCTCATCTTCAGATGGGTAGATTCGTTCATGCTCAGGTGTTCAGGCTTGGATT
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TCAGCTTATGCTCAGAACGGTGAGCCTATGGAGGCTCTTGAGATTTTTAGTCAGATGAGGAAGATG
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GGAAGCTATTGATATGTTTCATGAGATGATCAATAAAGATGTTAGACCCGACACCATCTCTATAAC
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GGAAGTGTAGAAGGCGCAAGGTTGGTTTTTGACCGAACACTTGACAGGGATGTTGTGGTGTGGAGT
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GAGCGTGGTGGAGTACATCCCAATGACGTCACTTTTCTGGGGCTTCTCATGGCCTGTAACCATTCAG
GCATGGTAAGAGAAGGCTGGTGGTTTTTCAACCGGATGGCGGATCACAAAATAAATCCACAACAG
CAACATTATGCATGTGTCATTGATCTTCTCGGGCGTGCTGGTCATCTGGATCAAGCTTATGAAGTGA
TCAAATGCATGCCGGTCCAGCCCGGTGTAACGGTTTGGGGAGCACTCCTAAGCGCTTGCAAGAAGC
ATCGCCACGTTGAACTGGGAGAATATGCAGCTCAACAGCTTTTCTCAATAGACCCATCCAACACAG
GCCACTATGTACAGCTCTCTAATCTGTATGCTGCAGCACGTTTGTGGGACCGTGTTGCAGAGGTGC
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TAGAGTGGATAGAGAGTAGATTGAAGGAAGGTGGATTTGTGGCTAACAAGGATGCTTCATTGCAC
GATCTCAACGATGAAGAAGCTGAAGAGACTTTGTGCAGCCACAGCGAGAGGATCGCGATTGCGTA
TGGGCTTATAAGTACACCCCAAGGCACACCACTGCGGATCACAAAGAATCTGAGAGCATGCGTAA
ACTGTCATGCTGCTACAAAGCTTATCTCGAAGCTAGTGGACAGAGAGATTGTTGTGAGAGATACAA
ATAGGTTCCACCATTTTAAGGATGGAGTTTGTTCCTGTGGTGATTATTGTCTTAAGGTTGAAAAGCT
TACTTATTTAATCATCAATTTAAAGATTCTGACAAACACAATAGAAAAATCTGTGAGATAAGCAAG
TCTATTTAAAATAATGAGAAGAAAGGAACATTGTCAAATAGAGTTGATTACTTCTCCCAGTTGGC

14.7. Sequence of the binary vector pAC106 including the T-DNA with marked Southern Blot probes

RB sequence: 5'-GACAGGATATATTGGCGGGTAAAC-3'

LB sequence: 5'-*CGGCAGGATATATTCAATTGTAA* -3'

Grey sequence: T-DNA

Unmarked sequence: Backbone of the binary vector pAC106

Bolded sequence: Southern Blot probe/LB/p106_f and p106_r

Blue marked sequence: Southern Blot probe/LB/LB fwd and LB rev

Pink marked sequence: Southern Blot probe/referred as RB probe, but located in the approximate middle of the T-DNA/pp1fwd and pp1 rev

GAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGaCTTGGCGTAAT
CATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGG
AAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTC
ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGG
GAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTT
CGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG
TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG
AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGC
TCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGC
TTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGT
GCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCC
GGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATG
TAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTG
GTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAC
AAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGAT
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GGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTT
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ACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCG
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CATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGG
TTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTAT
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AAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGA
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TGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAG
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AGGGTACCCGGGGATCAGATTGTCGTTTCCCGCCTTCGGTTTAAACTATCAGTGTTT**GACAGGATA**
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CGTATCCGATCGACGCCGATCAGCGCCTTGAAGGCCAAAGCCTGGAACTCACTTTGGGCACCGTTG
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CCTCCGCCGGCAACTGAAAGTTGACCCGCTTCATGGCTGGCGTGTCTGCCAGGCTGGCCAACGTTG
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AATGCGCTGCTTAACCAGCTCCACCAGGTCGGCGGTGGCCCATATGTCGTAAGGGCTTGGCTGCAC
CGGAATCAGCACGAAGTCGGCTGCCTTGATCGCGGACACAGCCAAGTCCGCCGCCTGGGGCGCTCC
GTCGATCACTACGAAGTCGCGCCGGCCGATGGCCTTCACGTCGCGGTCAATCGTCGGGCGGTCGAT
GCCGACAACGGTTAGCGGTTGATCTTCCCGCACGGCCGCCCAATCGCGGGCACTGCCCTGGGGATC
GGAATCGACTAACAGAACATCGGCCCCGGCGAGTTGCAGGGCGCGGGCTAGATGGGTTGCGATGG

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ACACGTACCCGGCCGCGATCATCTCCGCCTCGATCTCTTCGGTAATGAAAAACGGTTCGTCCTGGC
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GGTGGTCAAGCATCCTGGCCAGCTCCGGGCGGTCGCGCCTGGTGCCGGTGATCTTCTCGGAAAACA
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CGCGGGCATAGCCCAGCAGGCCAGCGGCGGCGCTCTTGTTCATGGCGTAATGTCTCCGGTTCTAGT
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TCCGTCGTATTTATAGGCGAAAGCAATAAACAAATTATTCTAATTCGGAAATCTTTATTTCGACGTG
TCTACATTCACGTCCAAATGGGGGCTTAGATGAGAAACTTCACGATCGATGCCTTGATTTCGCCATT
CCCAGATACCCATTTCATCTTCAGATTGGTCTGAGATTATGCGAAAATATACACTCATATACATAAA
TACTGACAGTTTGAGCTACCAATTCAGTGTAGCCCATTACCTTACATAATTCACTCAAATGCTAGGC
AGTCTGTCAACTCGGCGTCAATTTGTCGGCCACTATACGATAGTTGCGCAAATTTTCAAAGTCCTGG
CCTAACATCACACCTCTGTCGGCGGCGGGTCCCATTTGTGATAAATCCACCATCACAATAGATAGT
CTAATGGACGAAAAAGGCGAATATTTCGATGCTGAGATTCGACGCAATTAATTCGAGAAAAATCC
CGTGATTGATGCTGTTGAGTTACCAATAATATGGGCAGCGAAGGCCATTTAATTATAAGATCT
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TTCGCCAGTCTTTACGGCGAGTTCTGTTAGGTCCTCTATTTGAATCTTTG

GABI-Kat T-DNA binary vector (created by Bernd Reiss, sequenced by Mario Rosso / ADIS)

pAC106.seq Length: 10877 October 26, 2001 08:14 Type: N Check: 707

14.8. 1*N*—T-DNA—*CD tA* complete sequence

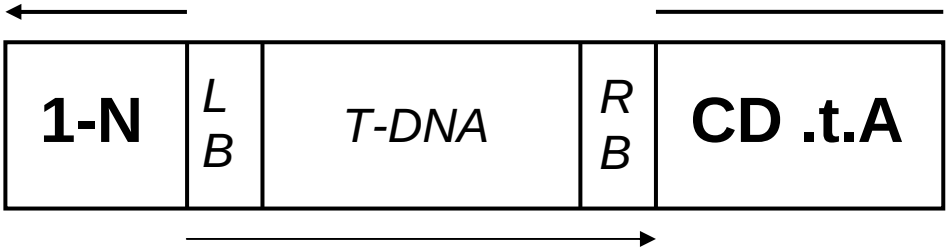

Fig. 66 AtDCN1 containing a T-DNA insertion

Yellow marked sequence: 3'-At3g12760-5'

Unmarked sequence: 5'-T-DNA insertion-3' within At3g12760

Blue marked sequence: Southern Blot probe/LB

Pink marked sequence: Northern Blot probe/referred as RB probe, but located in the approximate middle of the T-DNA

AGTCTTAAGGTTGAAAAGCTTACTTATTTAATCATCAATTTAAAGATTCTGACAAACACAATAGAA
AAATCTGTGAGATAAGCAAGTCTATTTAAAATAATGAGAAGAAAGGAACATTGTCAAATAGAGTT
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AGCAGAGGGAAAAAAACAAAAGGAGGAGAAGGAGAAAGCGCGTACCATAGTGAAGAAGAATCCA
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ATGCAAAAT

14.9. FST provided by GABI Kat

>83-K025604-022-814-C11-8409

AGCTACTGGAATTTTCAGGGGTATAAGAACAACTAGATACACGTTGTGCTATTGACCTTACATTGA
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TGATACCAACAATTGCTCTTCTCTCTGTTACCAATAATCACCACAGGAACAAACTCCATCCTTAAAA
TGGTGGAACCTATTTGTATCTCTCACAACAA

14.10. Alignment of *AtDCN*1 vs. FST

>lcl|31203
Length=498
Score = 652 bits (353), Expect = 0.0
Identities = 372/381 (97%), Gaps = 1/381 (0%)
Strand=Plus/Minus

```
Query  1    AGTCTTAAGGTTGAAAAGCTTACTTATTTAATCATCAATTTAAAGATTCTGACAAACACA
60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  AGTCTTAAGGTTGAAAAGCTTACTTATTTAATCATCAATTTAAAGATTCTGACAAACACA
322

Query  61   ATAGAAAAATCTGTGAGATAAGCAAGTCTATTTAAAATAATGAGAAGAAAGGAACATTGT
120
            |||||||||| ||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  321  ATAGAAAAATGTGTGAGATAAGCAAGTTTATTTAAAATAATGAGAAGAAAGGAACATTGT
262

Query  121  CAAATAGAGTTGATTACTTCTCCCAGTTGGCTGGTCACTTCTCAACGACATTTTTGTCAT
180
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Sbjct  261  CAAATAGAGTTGATTACTTCTCCCAGTGGGCTGGTCACTTCTCAACGACATTTTTGTCAT
202

Query  181  ACAAATACTCAACGAATTCATCAATCAGGTAAGGCCATGCTCCTTCTGCATCGTAATTCG
240
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Sbjct  201  ACAAATACTCAACGAATTCATCAATCAGGTAAGGCCATGCTCCTTCTGCATCGTAATTCG
142

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300
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
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82

Query  301  AGCCCATAGTTGCAATCAATGTAAGCTCAATAGCACAAC-TGTATCTAGTTTTTCTTTTA
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22

Query  360  CCCTTGAAAATTCCAGTAGCT  380
            ||| ||||||||||||||||
Sbjct  21   CCCCTGAAAATTCCAGTAGCT  1
```

14.11. Southern Blot probe/ RB

CATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTC
CACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTC
ACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAAT
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TATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAG
GAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATA

For detailed location of the probe within the T-DNA sequence, see also 14.7. and 14.8..

14.12. Southern Blot probe/ LB

GATTTCGCCATTCCCAGATACCCATTTCATCTTCAGATTGGTCTGAGATTATGCGAAAATATACACT
CATATACATAAATACTGACAGTTTGAGCTACCAATTCAGTGTAGCCCATTACCTTACATAATTCACT
CAAATGCTAGGCAGTCTGTCAACTCGGCGTCAATTTGTCGGCCACTATACGATAGTTGCGCAAATT
TTCAAAGTCCTGGCCTAACATCACACCTCTGTCGGCGGCGGGTCCCATTTGTGATAAATCCACCATC

ACAATAGATAGTCTAATGGACGAAAAAGGCGAATATTTCGATGCTGAGATTCGACGCAATTAATTC
GAGAAAAATCCCGTGATTGATGCTGTTGAGTTACCAATAATATGGGCAGCGAAGGCCATTTAATTA
TAAGATCTGATCCCCGGGT

For detailed location of the probe within the T-DNA sequence, see also 14.7. and 14.8..

15. MATERIALS

MS medium

4,4g/L MS
0,5g/L MES
10g/L sucrose
pH 5,7 (adjust pH 5,9-6; pH decreases during autoclaving)
6g/L Phytoagar (Duchefa Plant Agar)

CTAB buffer

	100mL
2% CTAB	2g
100mM Tris	1M pH8 10mL
1,4M NaCl	5M 28mL
20mM EDTA	0,5M 3,85mL
ddH_2O^a	7,95mL

fill up with ddH_2O^a to the proper volume mark → autoclaving
0,2% Mercapto-EtOH 200µL

Solutions for Southern blot

- 20x SSC (in autoclaved bottle, solution should be autoclaved)

In 1000mL ddH2O[a]
(3M) NaCl 175,3g
(3M) 3-Na-Dicitrate 88,2g
Fill up with ~750mL ddH2O[a]
Adjust pH to 7,0 with HCl/NaOH (only a few drops needed)

- 2x SSC/0,1%SDS

20x SSC 9mL
10% SDS 900µL
ddH_2O^a 80,1mL

- 1x SSC/0,1%SDS

20x SSC 4,5mL
10% SDS 900µL
ddH_2O^a 84,6mL

- 0,2x SSC/0,1%SDS

20x SSC 900µL
10% SDS 900µL
ddH_2O^a 88,2mL

- 0,1x SSC/0,1%SDS

20x SSC 450µL

10% SDS 900µL
ddH_2O[a] 88,6mL

- Neutralization solution (solution should be autoclaved)

In 1000mL ddH2O[a]
(1,5M) NaCl 87,65g
Tris-base 121,12g
0,5 M EDTA 2mL
Adjust pH to 7,4 (a lot of HCl needed, directly add 15mL HCl, ~30mL needed, check pH with pH paper)

- Denaturation solution (solution should be autoclaved)

In 1000mL ddH2O[a]
(1,5M) NaCl 87,65g
(0,5M) NaOH ~20g

- 0,25M HCl

987,45mL ddH2O[a] + 21,55mL 37% HCl

- Church Buffer (in autoclaved bottle, solution should be autoclaved)

ddH2O[a]		149,6mL
0,25M phosphate buffer	1M Na2HPO4	36mL
(72:28= Na2:Na)	1M NaH2PO4	14mL
(1mM EDTA)	0,5M EDTA pH 0,8	0,4mL
(1% BSA)	BSA*	2g (in fridge of cellculture room)
(7% SDS)	SDS**	14g (prepare 4x 3,5g portions)
	Sum:	200mL

* dissolve completely before adding SDS
** dissolve one portion completely before adding the other one

Solutions for Northern blot

- 10x MOPS Buffer for 1 L (use autoclaved bottle and ddH_2O[a] + DEPC)

MOPS 83,7g
NaAcetat ($3H_2O$) 13,6g; w/o H_2O 8,2g
EDTA (0,5M, pH8) 10mL
→ Adjust pH to 7,2 (NaOH)

- All other solutions used for Northern Blotting are prepared as Southern Blot solutions, except using ddH_2O[a] supplemented with DEPC.

Solutions for Western Blot

- Lacus Buffer (50 mL ddH_2O[a])

25mM Tris-HCl pH7,8	1,25mL 1M Tris-HCl
2mM MgCl2	14,93mg (- 6.H2O)
15mM EDTA	219,18mg
100mM NaCl	292,2 mg
10mM DTT	77,125mg

0,1% TWEEN-20	50µL
0,5mM PMSF	4,355mg = 0,004355g

- 10x TB Buffer (1000mL ddH_2O[a])

50mM Tris	6,075g
50mM Boric acid	3,09g

- 10x SDS Running Buffer (500mL ddH_2O[a])

250mM Tris	15,143g
1,92M Glycine	72,06g
1% SDS	1,442g

- 10x PBS (50 mL ddH_2O[a])

1,4M NaCl	40,908g
27mM KCl	1g
100mM Na2HPO4	17,907g
18mM KH2PO4	1,22g

- 10x Running Buffer (1000mL ddH_2O[a])

192mM Glycin	144g
25mM Tris	30g Tris-Base
0,1% SDS	10g

- SDS-PAGE/Gels for Western Blot Analysis

	separating gel			**stacking gel**	
	3,5mL	7mL		1mL	2mL
	12,50%	12,50%	10%		
40% Acrylamid/ 0,8% Bisaycrylamid [mL]	1,094	2,188	1,75	0,125	0,25
1M TrisHCl pH 8,8 [mL]	1,313	2,625	2,625		
1M TrisHCl pH 6,8 [mL]				0,125	0,25
dH2O [mL]	1,0275	2,055	2,489	0,734	1,468
10% SDS [µL]	35	70	70	10	20
10% APS [µL]	30	60	60	10	20
TEMED [µL]	3	6	6	2	4

Buffer for pollen collection

(1000mL ddH_2O[a])
50mM $NaPO_4$ 5,9g
1mM EDTA 372,24mg/ 0,37224g
0,1% Triton X-100 one drop

16. APPENDIX

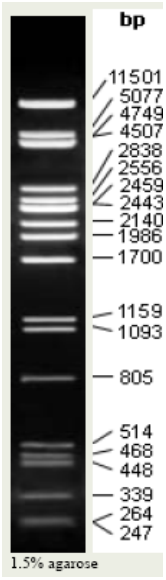

Fig. 67 Lamda/pst length marker[8]

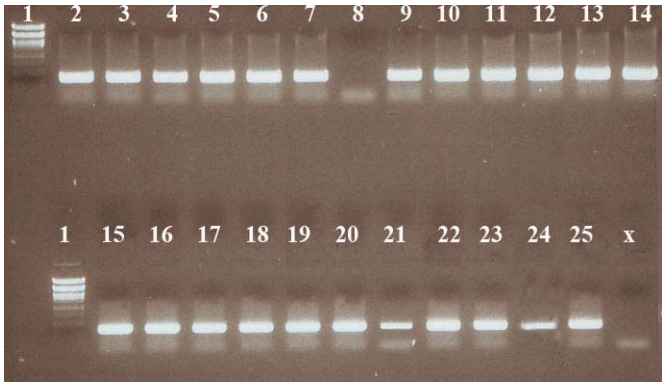

Fig. 68 cDNA synthesis check via PCR with 18s primer/BX2a/BX3 and BX3a

For RNA isolation of BX1a and BX2a plants, material from leaves was isolated and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were separated on an 1% agarose gel and visualized by ethidium bromide staining.

Lane 1: Lambda/pst marker
Lane 2: wt col-O
Lane 3: 2A/2e
Lane 4: 2B/3c
Lane 5: 2C/3d
Lane 6: 2D/3c
Lane 7: 3A/2a
Lane 8: x
Lane 9: 4A/1d
Lane 10: 4B/2d
Lane 11: 4D/2b
Lane 12: 5B/2b
Lane 13: 5C/2e
Lane 14: 5D/1d
Lane 15: wt col-O
Lane 16: 814C11/24-5-11 x wt b
Lane 17: 814C11/24-5-1 x wt 1a
Lane 18: 814C11/24-5-1 x wt 2a
Lane 19: 814C11/24- 10-10 x wt b
Lane 20: 814C11/24-10-6 x wt a
Lane 21: 814C11/24-6-10 x wt A4
Lane 22: 814C11/24-6-10 x wt A4
Lane 23: 814C11/24-6-3 x wt B3
Lane 24: 814C11/24-6-10 x wt B4
Lane 25: **814C11/24**-6-3 x wt C2
Lane x: Negaitve Control

[8] http://www.imbb.forth.gr/groups/minotech-new/pdf/lambda_DNA_PstI_digest.pdf

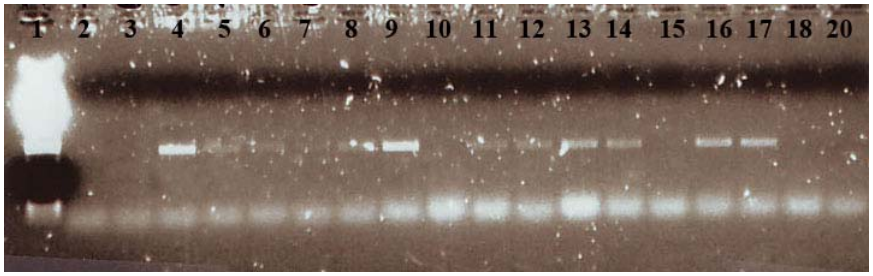

Fig. 69 Expression Analysis via PCR with At3g12760 primers/all BXn and Bxna/2nd attempt
For RNA isolation of BX1a and BX2a plants, material from leaves was isolated and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were separated on an 1% agarose gel and visualized by ethidium bromide staining.

Lane 1: Lambda/pst marker
Lane 2: 3A/2a
Lane 3: 4A/1d
Lane 4: 4B/2d
Lane 5: 4D/2b
Lane 6: 5B/2b
Lane 7: 5C/2e
Lane 8: 5D/1d
Lane 9: wt col-O
Lane 10: **814C11/24**-5-11 x wt b
Lane 11: **814C11/24**-5-1 x wt 1a
Lane 12: **814C11/24**-5-1 x wt 2a
Lane 13: **814C11/24**-10-10 x wt b
Lane 14: **814C11/24**-10-6 x wt a
Lane 15: **814C11/24-** 6-10 x wt A4
Lane 16: **814C11/24**-6-3 x wt B3
Lane 17: **814C11/24**-6-10 x wt B4
Lane 18: **814C11/24**-6-3 x wt C2
Lane 20: Negative Control

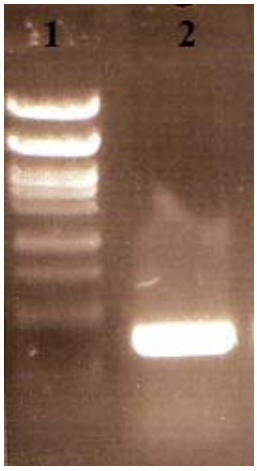

Fig. 70 Northern probe cDNA/18s
For RNA isolation material was isolated from leaves and checked via PCR on cDNA level, as described in Materials and Methods. The PCR products were separated on an 1% agarose gel and visualized by ethidium bromide staining. Lane 1 represents length marker Lambda/pst, while lane 2 shows one band 1022bp in size amplified from wild type cDNA with 18s primers and purified using Wizard SV gel and PCR purification system.

17. CURRICULUM VITAE

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1990-1994 Primary school,Volksschule VHS 9, Körner Schule, Klagenfurt, Carinthia, Austria

1994-2000 Secondary grammar and partially secondary upper school Sport-Realgymnasium, Lerchenfeldstrasse, Klagenfurt, Carinthia, Austria

2000-2002 Secondary upper school Bachmanngymnasium, Klagenfurt, Carinthia, Austria

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Major subjects: Mathematics, german, english deepening
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Universitary Education

October 2002-December 2007 Study of Biology with emphasis on Genetics and Microbiology at the University of Vienna, Austria

Major subject: Cell Biology, Immunology
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January-February 2008 „Vertiefungsübungen“ in the laboratory of A.o. Prof. Alisher TOURAEV, Dipl. Ing. Dr. Alexandra Ribarits Max F. Perutz Labors, Department für Pflanzenmolekularbiologie University, Vienna, Austria

March 2008- March 2009 Diploma Thesis “Molecular characterization and analysis of a putative *AtDCN*1 T-DNA knockout lines”

Supervision of students

January-March 2009 Supervision of Tina Hofmaier during her “Vertiefungsübungen”

Additional Scientific Working Experience

January-September 2007 *Institut für Molekulare und Medizinische Diagnostik, AKH, Vienna, Austria*

Participated Conferences

February 2008 “Molecular Mapping & Marker Assisted Selection in Plants”, Vienna, Austria

September 2008 "1st international PhD school-Plant Development", Retzbach, Germany
February 2009 "Plant abiotic stress tolerance", Vienna, Austria

Poster
September 2008 "*NtDCN*1 is involved in phase changes during gametophytic development and embryogenesis"
Julia Hosp, Alexandra Ribartis, Katarzyna Szaszka, Alisher Tashpulatov, Tatiana Resch, Jin Yongfeng, Christina Friedmann, Viktor Voronin, Elisabeth Ankele, Irina Sadovnik, Erwin Heberle-Bors and Alisher Touraev
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Languages German (native language)
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18. LEBENSLAUF

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Schulausbildung
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2000-2002 Bachmanngymnasium, Klagenfurt, Kärnten, Österreich

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Hauptfächern: Mathematik, Deutsch, English vertiefend
Nebenfächern: Biologie, Geographie, Philosophie und Psychologie

Universitäre Ausbildung
Oktober 2002-Dezember 2007 Studium der Genetik und Mikrobiologie an der Universität Wien, Österreich
- Nebenfächer: Zellbiologie und Immunologie
- Hauptfach: Cyto- und Entwicklungsgenetik

WS und SS des Jahres 2006/2007 wurde mit einem Notendurchschnitt von 1,1 absolviert

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Betreuung von Studenten

Januar-Februar 2009	Betreuung von Tina Hofmaier während ihrer Vertiefungsübungen

Zusätzliche wissenschaftliche Arbeitserfahrungen

Januar-September 2007	I*nstitut für Molekulare und Medizinische Diagnostik, AKH, Wien, Österreich*

Teilgenommene Konferenzen

Februar 2008	"Molecular Mapping & Marker Assisted Selection in Plants", Wien, Österreich
September 2008	„1st international PhD school-Plant Development", Retzbach, Deutschland
Februar 2009	"Plant abiotic stress tolerance", Wien, Österreich

Poster

September 2008	"*NtDCN*1 is involved in phase changes during gametophytic development and embryogenesis" Julia Hosp, Alexandra Ribartis, Katarzyna Szaszka, Alisher Tashpulatov, Tatiana Resch, Jin Yongfeng, Christina Friedmann, Viktor Voronin, Elisabeth Ankele, Irina Sadovnik, Erwin Heberle-Bors and Alisher Touraev 1st international PhD school-plant development, Retzbach, Deutschland

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