Title: A Tobacco Homolog of DCN1 is Involved in Cellular Reprogramming and in Developmental Transitions

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SUMMARY

Plant proteomes show remarkable plasticity in reaction to environmental challenges and during developmental transitions. Some of this adaptability comes from ubiquitin-mediated protein destruction regulated by cullin-RING E3 ubiquitin ligases (CRLs). CRLs are activated through modification of the cullin subunit with the ubiquitin-like protein RUB/NEDD8 by an E3 ligase called DEFECTIVE IN CULLIN NEDDYLATION 1 (DCN1). Here we show that tobacco DCN1 binds ubiquitin and RUB/NEDD8, and associates with cullin. When knocked down by RNAi, tobacco pollen formation stopped and zygotic embryogenesis was blocked around the globular stage. Additionally we found that RNAi of DCN1 inhibited the stress-triggered reprogramming of cultured microspores from their intrinsic gametophytic mode of development to an embryogenic state. This stress-induced developmental switch is a known feature in many important crops and leads ultimately to the formation of haploid embryos and plants. Compensating the RNAi effect by retransformation with a promoter-silencing construct restored pollen development and zygotic embryogenesis, as well as the ability for stress-induced formation of embryogenic microspores. Overexpression of DCN1, however, accelerated pollen tube growth and increased the potential for microspore reprogramming. These results demonstrate that the biochemical function of DCN1 is conserved in plants and that its activity is specifically required for transitions during pollen development and embryogenesis, and for pollen tube tip growth.
INTRODUCTION

Plant reproduction is characterized by a number of important developmental transitions, beginning with the acquisition of polarity in the globular embryo [1]. The most fundamental transition in the life cycle of higher plants is the alternation of generations in which diploid sporophytes produce haploid male and female gametophytes [2], [3]. Male microgametogenesis leads to the formation of pollen, whereas female megagametogenesis produces the embryo sac enclosing the egg cell [4].

Cultured microspores remarkably show both developmental plasticity and high adaptability to stress [5]. Diverse stress treatments, such as temperature shock, nutrient starvation, or chemicals, abolish the gametophytic program intrinsic to microspores [6]. Instead of developing into pollen, stressed microspores become totipotent and develop into haploid embryos and plants under non-stress conditions [7]. This process corresponds to a fundamental developmental process in plants, i.e. the transition of a gametophyte into a sporophyte, but can be seen also as the reprogramming of a cell with a restricted development fate to a totipotent state. Homozygous doubled haploids emerging after spontaneous or induced chromosome doubling in the regenerated haploids are both scientifically attractive and highly valuable for plant breeding [7], [8]. However, knowledge about the molecular mechanisms controlling the developmental switch of cultured microspores towards microspore embryogenesis is still fragmentary.

The ubiquitin proteasome pathway, active in all eukaryotes, mediates protein degradation through sequential enzymatic action of a ubiquitin-activating enzyme E1, a ubiquitin-conjugating protein E2, and a substrate-specific ubiquitin E3 ligase (reviewed by [9]). The largest class of E3 ligases, cullin RING ligases or CRLs, are multi-subunit protein complexes. The cullin subunit acts as platform protein, a RING H2 finger protein mediates contact to an E2 protein, and variable substrate-recognition subunits (SRS) such as F-box proteins are connected to the complex by adaptors (reviewed by [10], [11]). Targeted protein degradation by CRLs has been associated with photomorphogenesis, pollen recognition by the stigma, pathogen response, and the response to various plant hormones (reviewed by [12], [13]), including auxin (reviewed by [14], [15]).

CRL activity is controlled via modification of the cullin subunit by the ubiquitin-like protein RUB/NEDD8 in fission yeast and animals. The Arabidopsis genome contains three RUB/NEDD8 genes, two of which, RUB1 and RUB2, regulate diverse processes throughout plant development and are essential as demonstrated by embryo lethality in rub1 rub2 double mutants [16], [17]. Derubylation is accomplished by the COP9 signalosome [18] whereas
rubylation is mediated by an enzymatic cascade, which, analogous to ubiquitylation, comprises three different enzymes (E1, E2, E3). In Arabidopsis, the RUB E1 and E2 functions are performed by AXR1/ECR1 and RCE1, respectively [19], [20]. Rubylation/neddylation and derubylation/deneddylation have been shown to be essential in plants, worms, and mammals, partially in fission yeast, but not in budding yeast (reviewed by [9], [21]). They play a fundamental role in important processes such as morphogenesis [22], cell division [23], signaling [24] and embryogenesis [25],[26].

A crucial break-through in understanding the regulation of cullin rubylation, and hence ubiquitin ligase control, was the discovery of DEFECTIVE IN CULLIN NEDDYLYATION 1 (DCN1) in worm and yeast [26]. Acting as a scaffold-like RUB/NEDD8 E3 ligase in association with the RING H2 finger protein RBX1 [27], DCN1/Dcn1p was proven to be required and sufficient for cullin neddylation \textit{in vitro} and \textit{in vivo} [26],[28]. Recent X-ray crystal structure analysis established that yeast DCN1 contains an N-terminal ubiquitin-binding (ubiquitin-associated or UBA) domain and a large C-terminal POTENTIATING NEDDYLYATION (PONY) domain, that binds NEDD8 and provides the surface for interaction with cullins and the RUB/NEDD8 E2 enzyme Ubc12 [28]. Since most components of the ubiquitin-proteasome pathway are conserved throughout the eukaryotic kingdoms [29], the existence of a DCN1 homolog in plant genomes comes as no surprise.

By using suppression subtractive hybridization of stressed embryogenic and non-stressed gametophytic microspores we previously reported several differentially expressed genes [30]. One of them, ntsm10 (Nicotiana tabacum stressed microspore 10), was found to be a homolog of nematode DCN-1, yeast Dcn1p, and human SSCRO [31], and accordingly renamed into Nt DCN1 (Nicotiana tabacum DCN1). Since DCN1 properties and function in plants remained to be analyzed, we used biochemical binding assays, RNAi and overexpression to investigate its function during developmental transitions in tobacco. Here, we show that plant DCN1 binds ubiquitin and RUB/NEDD8 similar to DCN1 orthologs from other eukaryotes, and physically associates with cullin. Overexpression of DCN1 in tobacco promotes the formation of embryogenic microspores and accelerates pollen tube growth. Using RNAi knockdown we show that DCN1 controls the key developmental transitions from microsporogenesis to microgametogenesis, from gametophytic to sporophytic development in cultured microspores, and from the globular to the heart-shaped stage in both zygotic and microspore embryogenesis.
RESULTS

A Tobacco DCN1 Ortholog Shows Conserved Ubiquitin and RUB/NEDD8 Binding, and Associates with Cullin in Vitro

Nt DCN1 encodes a 30-kDa protein comprising 259 amino acids and is represented by two almost identical paralogs in the allotetraploid tobacco genome - Nt DCN1A (DQ885939) and Nt DCN1B (FJ976682) (Figure 1a). Nt DCN1 has high similarity to nematode DCN-1 and yeast Dcn1p and contains UBA and PONY domains [26]. The closest homolog of Nt DCN1 in Arabidopsis is an unknown protein (At3g12760) with 78% identity and similar domain architecture. Another close relative, At3g28970 (ANTI-AUXIN-RESISTENT 3, AAR3) with 45% identity but without a UBA domain, has recently been shown to regulate root responses to 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin [32]. A third, still uncharacterized PONY domain-containing protein, At1g15860, shares 32% identity with Nt DCN1.

To demonstrate the functional properties of DCN1 we tested its ability to bind ubiquitin and RUB/NEDD8 in vitro. A DCN1-specific polyclonal antibody detected the expected 30-kD DCN1 protein on Western blots of eluted fractions resulting from incubation of recombinant DCN1 with ubiquitin and RUB/NEDD8 beads, respectively (Figure 1b). Thus, Nt DCN1 readily binds ubiquitin and RUB/NEDD8, similar to yeast DCN1p and nematode DCN-1 [26]. A pull-down experiment using At CUL1 shows that DCN1 associates with cullin (Figure 1c and Supporting Information Fig. 1), confirming data obtained in yeast and nematodes [26], [28].

Nt DCN1 is Expressed Throughout the Plant Body, and Expression is Differentially Regulated During Pollen Development and in Reprogrammed Cultured Microspores

Histochemical analyses of transgenic tobacco lines harboring a DCN1pro::GUS construct demonstrated promoter activity throughout the plant body and indicated developmental regulation. In mature plants, the major site of GUS expression was the vascular system, similar to seedlings (Figure 2a). Strong GUS signals were also detected in all meristematic and actively developing regions (Figure 2b-e). The DCN1 promoter was also active in reproductive tissues, as observed in axile placentas of unfertilized ovaries (Figure 2f) and in mature pollen (Figure 3e). Weak GUS expression was found in early embryos and endosperms (Figure 2g), visualized in dark-field images demonstrating mostly spotted GUS expression in these tissues (Figure 2h). Mature seeds showed spotted GUS signals and strong
GUS expression was observed at the embryonic root tip upon imbibition (Figure 2j-l). Early seedlings had GUS signals in cotyledons and the distal part of the hypocotyl (Figure 2l). Northern blot analyses confirmed that DCN1 transcripts are present in different organs of tobacco plants, with higher expression levels in young tissues (Figure 3a).

Both RNA and protein blots showed that DCN1 is present throughout pollen development and microspore embryogenesis, in accordance with DCN1 promoter activity (Figure 3b,c,e). DCN1 transcript levels were detected with the 3'-UTR of DCN1, which recognizes both DCN1 paralogs. They were high in unicellular microspores but decreased after the first pollen mitosis, i.e. in bicellular and mature pollen (Figure 3b). Protein levels, in contrast, increased strongly during pollen development from unicellular microspores to mature pollen (Figure 3c).

A slight increase in expression was detected on RNA blots between freshly isolated microspores and cultured microspores subjected to a stress treatment for six days for reprogramming into embryonic development (Figure 3b), similar to protein levels (Figure 3c). An increase in expression was detected also by in situ hybridization, by GUS assays in DCN1pro::GUS plants, (Figure 3d,e), and by RT-PCR (Figure 5b). DCN1 transcripts, promoter activity, and DCN1 protein were consistently detected in 25-day-old microspore-derived embryos (Figure 3b-e) while a reduction of DCN1 expression was observed in 50-day-old, late torpedo-shaped embryos on the protein level (Figure 3c) and at the cotyledon stage on the RNA level (Figure 3d).

**RNAi of Nt DCN1 Leads to Severe Impediment of Tobacco Pollen Development, Embryogenesis, and Changes in Cullin Rubylation**

RNAi was used to study the in vivo function of DCN1. A 300-bp 3’ UTR fragment corresponding to the originally identified SSH sequence was cloned into a vector downstream of the constitutive CaMV 35S promoter in sense and anti-sense orientations linked by an intron [33]. Reductions in mRNA and protein levels, as observed in young leaves, varied from weak to strong in individual lines, as expected for RNAi knock-down plants. Both DCN1 mRNA levels and DCN1 protein levels were strongly reduced in leaves of RNAi lines (Figure 4a). This indicated that due to their high sequence similarity both DCN1-paralogs were knocked down by RNA interference.

Nine RNAi lines with strong and six lines with medium reduction of DCN1 transcript abundance were analyzed for pollen and seed development. Plants with a strong reduction in RNA and protein levels were in general smaller compared to wild-type plants, including...
flowers, but did not show any other morphological changes. Seed pods contained mainly stunted seeds of different sizes. Mature anthers of primary RNAi lines enclosed varying numbers of dead pollen plus mature pollen in frequencies ranging from 5-40%, which correlated with the degree of DCN1 down-regulation.

To analyze the effect of a DCN1 knock-down on reproductive development, homozygous RNAi lines were produced by selfing and kanamycin selection of seeds. Homozygosity was verified by non-segregation of the kanamycin gene in the F2 progeny, and Northern and Western blots confirmed the transmission of the RNAi effect in leaves and microspores (Figure 4a, Supporting Information Fig. 2). Three independent homozygous lines (L25, L35 and L47) were selected for further phenotypic analyses. The viability of microspores and early bicellular pollen grains were lower than in wild-type plants (Table 1) whereas the majority of pollen grains appeared to be dead (Figure 3b, L47, Table 1). Consequently, the majority of seeds were stunted, both after self-pollination and after backcrossing with wild-type pollen, indicating reduced female fertility. Seeds were arrested at different stages, mainly at the pre-globular to globular stages (Figure 4c,d).

Microspores isolated from those plants and cultured in a pollen maturation medium [34], [35] developed into mature pollen at frequencies similar to microspores developing in anthers on the plants (Supporting Information Table 1), demonstrating that DCN1 expression was knocked down within the microspores (gametophytic effect) rather than within the surrounding anther tissues (sporophytic effect).

To study the role of DCN1 in microspore embryogenesis, unicellular microspores were isolated before pollen mitosis I (PM I), i.e. prior to their in vivo death, from anthers of homozygous RNAi lines. These and corresponding wild-type microspores were subjected to a mild heat shock at 33°C and starvation in a sugar and nitrogen-free medium for six days [35]. In the wild-type, this stress treatment resulted in the formation of embryogenic microspores, and the formation of embryos (Figure 4e) and haploid plants. In contrast, although originally viable, the vast majority of microspores isolated from all three homozygous RNAi lines died during the stress treatment. However, a few microspores (approx. 2% of the total population) survived and dedifferentiated into embryogenic microspores. When enriched by Percoll gradient centrifugation and cultured in embryogenesis medium, these embryogenic microspores were able to form embryos. But, similar to zygotic embryos, they were either arrested at around the globular stage with irregular shapes or strongly delayed in their further development (Figure 4e).
Alleviation of Nt DCN1 RNAi by Transcriptional Gene Silencing Reconstitutes the Wild-Type Phenotype

To prove that the observed phenotypes were caused by DCN1 knock-down, we used transcriptional silencing of the promoter controlling the RNAi construct to restore the wild-type phenotype. The construct used for re-transformation contained an inverted repeat of the full 35S promoter under control of the constitutively active ubiquitin promoter and lacked a poly-adenylation site, thereby switching off the 35S promoter by RNA-directed DNA methylation [36], [37]. The HPT (hygromycin phosphotransferase) gene was included in the construct to be able to select double transformants on hygromycin and kanamycin. Both DCN1 mRNA and protein levels recovered in the double-transgenic lines, exemplified by line PS-L47 in Figure 4a, and hyper-methylation of the 35S promoter was confirmed (Supporting Information Fig. 3). As anticipated, plant and flower size, pollen development, seed set, and zygotic embryogenesis, as well as microspore embryogenesis, were restored with frequencies similar to wild-type plants in all lines with reconstituted DCN1 expression. As representative example reconstitution is shown for PS-L47 (Figure 4b,e). We thus conclude that the phenotypes seen in homozygous DCN1 RNAi lines were indeed caused by a knock-down of the DCN1 gene.

Overexpression of Nt DCN1 Accelerates Pollen Tube Growth and Promotes Reprogramming of Microspores

Transgenic plants were created that harbored the DCN1 full-length cDNA under control of the DC3 promoter, shown to be highly active during male gametophyte development and microspore embryogenesis [38], [39]. Out of 25 independent transgenic lines overexpressing DCN1 in young leaves, two lines with strong overexpression in microspores (ox14 and ox21, 4A, B) were self-pollinated, and homozygous offspring were produced. 

Pollen viability and germination, as measured by FDA staining and in vitro germination test, did not differ much between wild-type plants and overexpressor lines (Table 2) and the difference between overexpressor lines depended on the degree of overexpression. Significant differences, however, were detected in pollen tube growth rates (Figure 5d). With 10.22 µm/min for ox14 and 7.95 µm/min for ox21, overexpressor pollen tubes in GK-medium grew at about twice the speed of wild-type pollen tubes (4.38 µm/min, Table 2). Wild-type tobacco pollen, in turn, grew at more than double the speed reported by [40], probably due to a more appropriate culture medium, while ox14 pollen tube growth rates approached in vivo growth rates of wild-type tobacco pollen (25 µm/min, [41]).
To evaluate whether DCN1 is not only necessary but sufficient for stress-induced reprogramming of microspores towards embryogenesis, we incubated microspores from wild-type and overexpressor plants under reprogramming conditions, followed by culturing them in embryogenesis medium for embryo formation. DCN1 transcript levels, measured throughout the six-day stress treatment, during which reprogramming occurs in the wild-type, increased continuously in both wild-type and DCN1-overexpressing microspores, and clearly higher levels were detected in overexpressing microspores already during the first days of culture (Figure 4b). Approximately 80% of wild-type microspores dedifferentiated into embryogenic microspores after a stress treatment of six days (Table 3), similar to published data [42]. After two weeks in embryogenesis medium most of the reprogrammed microspores had developed into multi-cellular structures, and after six weeks, these had formed well-developed globular to torpedo-shaped embryos. A shorter, two-day, exposure to the stress treatment decreased the frequency of embryogenic microspores to 40%, and only 10-12% of the surviving cells developed into multicellular structures and embryos (Table 3, Figure 5c). In the overexpressor lines, the frequency of embryogenic microspores after two days of stress treatment reached almost 70%, similar to wild-type microspores after a six-day stress treatment, and the majority of these embryogenic microspores developed into multicellular structures and embryos (Table 3, Figure 5c).

When we completely omitted the stress treatment and cultured ox14 and ox21 microspores directly in embryogenesis medium, no embryos formed. Cytological investigations of embryogenic cultures, however, revealed that microspores from both overexpressor lines developed into multi-cellular structures faster and at a higher frequency than wild-type microspores (Table 4).

**DISCUSSION**

**Nt DCN1 Shares Biochemical Features with Other Eukaryotic DCN1 Proteins**

We have found that Nt DCN1 binds cullin, ubiquitin, and RUB, similar to DCN1 proteins of other eukaryotes and consistent with the features of a potential RUB E3 ligase [28]. Ubiquitin binding is particular to DCN1 and distinguishes the DCN1 gene from DCN1-like genes such as AAR3, the only member of the DCN gene family characterized in plants to date [32]. Unfortunately, due to the lack of suitable cullin and RUB antibodies, we were not able to show differences in cullin rubylation. Further experiments will have to show whether cullins are DCN1-mediated rubylation targets in the developmental transitions that we have
described, and if so, which specific CRLs they constitute. These could be SCF-like complexes similar to those involved in cell cycle regulation [23], [43], [44] or in the signal transduction of hormones such as auxin, gibberellins, abscisic acid, brassinosteroids, jasmonic acid, and ethylene [45], [13].

In addition, we expect non-cullin proteins to be rubylated, as the list of neddylated proteins is increasing [28], [21]. In mammals, the E3 ligase Mdm2 mediates neddylation of p53 [46], [47], leading to negative regulation of its transcriptional activity [48]. Neddylation of tyrosine receptor kinases by the dual-function c-Clb E3 ligase leads to receptor recycling and signal attenuation [49]. It would therefore not be surprising if plant DCN1 proteins, too, rubylated non-cullin substrates.

Expression of Nt DCN1 Overlaps with Other Rubylation Genes and Points Towards Auxin-Mediated Regulation

DCN1 is expressed throughout the plant body, particularly in actively developing tissues and organs which are sites of developmental transitions such as shoot and root tips, lateral root initials, lateral shoot buds, female and male gametophytes, embryos, and seeds. In many respects, the expression of Nt DCN1 is similar to that of AXR1 and ECR1, the two components of a RUB E1, and of RCE1, encoding the E2 for rubylation in Arabidopsis [19], [20]. Mutants of axr1, ecr, and rce1 have a seedling-lethal phenotype characteristic for a defect in auxin signaling [19], [20], [50]. These mutants are smaller than wild-type plants and have smaller flowers, similar to what we observed in DCN1 knock-down lines. The plant growth regulator auxin is important in many aspects of plant development, including processes that have been distorted in our DCN1-lacking plants, such as embryogenesis [1] as well as anther and pollen development [51]. A DCN1-like gene, namely AAR3, lacking the N-terminal UBA-domain present in all known eukaryotic DCN1 orthologs compared so far, has recently been identified through an auxin-resistant mutant screen in Arabidopsis [32]. However, no evidence for its participation in Aux/IAA protein degradation has been reported, and the phenotype described for the aar3 mutant shows little similarity with that of our Nt DCN1 knock-down lines.

Nt DCN1 is Necessary for Pollen Development while Overexpression Promotes Pollen Tube Growth

During pollen development, DCN1 transcript levels quickly decreased after PM I and remained low in mature pollen grains, similar to the expression of its Arabidopsis ortholog
At3g12760 [52], while protein levels strongly increased. This phenomenon has been described for a large number of pollen-expressed genes [53], [54]. A feedback mechanism may be operating in developing pollen grains, down-regulating DCNI transcription when the protein has accumulated beyond a certain threshold level.

A loss of function of DCNI in pollen, induced by RNAi-mediated knock-down, led to a developmental arrest after PM I in early bicellular pollen. This stage marks the developmental phase change from microsporogenesis to microgametogenesis, i.e. when genes required for microgametogenesis are activated after PM I [55]. Pollen defects have also been found in other components of the rubylation and ubiquitylation pathways. Pollen of insertion mutants in the DNC1-like AAR3 gene failed to transmit the auxin-resistant phenotype to the next generation when used to pollinate wild-type Arabidopsis plants [32]. Similarly, mutants in the AXR1 gene, encoding a RUB E1 [19], produced significantly less pollen [56], and also phytotoxin-resistant mutants in CORONATINE-INSENSITIVE 1 (COI1), which is part of an SCF$^{COI1}$-complex involved in jasmonate signaling [57], had a male sterility phenotype [58].

Pollen lethality has been observed in Arabidopsis thaliana RNAi plants to be unrelated to the loss of function of particular genes but to be a general feature of RNAi knock-down in this species [59]. Unlike in Arabidopsis, however, the frequencies of dead pollen was much higher in our tobacco RNAi knock-down plants, microspores were shown to be normal, and we observed defects not only in pollen but also in seed set and embryonic development. In addition, our experiments to alleviate the RNAi knock-down of Nt DCNI by transcriptional silencing of the 35S-promoter driving the Nt DCNI RNAi construct showed that complementation restored all those functions affected by RNAi knock-down, i.e. pollen lethality as well as zygotic and microspore embryogenesis, arguing for a specific effect of RNAi on Nt DCNI. Thus, it seems that tobacco is better suited for RNAi knock-down experiments than Arabidopsis, at least concerning pollen development.

In DCNI overexpressor lines, pollen development per se was not significantly affected, presumably as a consequence of the already high amount of DCN1 protein present in mature wild-type pollen. DCN1 thus is stored in mature pollen, presumably to be used during pollen germination and tube growth. It is likely that a threshold level of DCN1 is required to support the timely post-mitotic regulation of protein degradation through its anticipated role in rubylation. In a similar mechanism, the ubiquitin RING-type E3 ligases RHF1a and RHF2a control pollen development through post-meiotic ubiquitylation and subsequent degradation through the 26S proteasome [60], [61].
Overexpression of DCN1 resulted in a clear acceleration of pollen tube growth compared to the wild-type, possibly due to the increased degradation of stored proteins. Proteome analyses have verified that pollen grains store synthesized proteins for later use [53], [62], [63]. Pollen function appears to depend on selective protein degradation by the 26S proteasome [64], [63]. Indeed, the proteolytic machinery is required during virtually all stages of pollen development and germination [65], [66], [67], [68], including the control of self-incompatibility [69]. Our results suggest that DCN1 is an important player in these processes and that rubylation may activate ubiquitin E3 ligases specific for repressor proteins, e.g. such controlling translation of stored mRNAs, or specific for ubiquitin E3 ligase growth factor receptors regulating pollen tube growth. Auxin is well known to stimulate pollen tube growth [70] and pollen contains proteins involved in auxin signal transduction [71].

**Nt DCN1 is Necessary for Zygotic Embryogenesis**

DCN1 is required for embryogenesis. The developmental arrest at the globular stage of embryogenesis found in DCN1 RNAi lines resembled that in Arabidopsis cullin3 mutants [72], [73]. A similar phenotype was found in apc2 mutants [60]. APC2 is a distant member of the cullin protein family and encodes for a subunit of the E3 ligase APC/C (anaphase-promoting complex or cyclosome). Furthermore, in silico expression data of At3g12760 (At DCN1) and cullins indicate their close correlation in diverse physiological contexts, and we predict a tight functional connection between DCN1 and the cullin rubylation pathway similar to worm and yeast.

In early plant embryogenesis, new cell fates are established with virtually every round of mitosis [1]. Targeted protein degradation by CRLs is required not only for the precise timing and positioning of cell divisions [74], [75], but also for the action of auxin [76], [77], [78], and possibly other hormones that signal through CRLs and regulate embryogenesis [45], [13]. Thus, various CRLs may be subject to cullin neddylation, and DCN1 may have a large pleiotropic effect on embryogenesis.

Although both male and female reproductive development were affected in the RNAi lines, the RNAi trait was transmitted to the offspring through a few functional gametes, indicating that DCN1 knock-down may have been incomplete. Our results show that DCN1 is an essential gene, but that its residual expression in the RNAi knock-down plants was sufficient to maintain vegetative processes and allowed pollen formation and seed development, though at a significantly reduced rate. Although residual DCN1 expression
may have been the cause of incomplete penetrance and variable expressivity, another possible explanation is that a basal level of rubylation is retained in the absence of DCN1. Indeed, cullin neddylation has been shown to occur without DCN1 activity in vitro through direct neddylation of cullins by RBX1, albeit less efficiently [26], [27]. Recent data, in addition, have shown that DCN1 is sufficient for neddylation in a purified recombinant system [28]. In nematodes, RNAi-mediated knock-down of DCN1 caused severe spindle defects and a complete developmental arrest in embryos, proving that DCN1 is an essential gene [26]. On the other hand, in the same study it was established that Dcn1p is not essential in budding yeast. In line with our data, incomplete penetrance and variable expressivity have been reported in Arabidopsis cul1 and cul3 mutants [79], [72], [73] and the rhf1a rhf2a (RING-H2 group F) RING-type E3 ligase double mutant [61], in which male and female development were affected in different developmental stages. Only a fraction of embryo sacs, pollen, and embryos aborted while other embryos with a mutated gene developed normally. Thus, the formation of functional pollen and seeds cannot solely be attributed to an incomplete knock-down by RNAi, because, taken together, these results indicate that the diverse E3 ligase components are of varying importance during reproductive development. Other components of the neddylation/rubylation pathway, namely RUB/NEDD8 E1 and E2, are essential in both animals and plants [9], [11].

Nt DCN1 is Necessary and Increases the Potential for the Reprogramming of Microspores Towards Totipotency

Despite its scientific attractiveness and the economic importance of microspore-derived doubled haploid plants for plant breeding [8], the molecular mechanisms controlling this process have remained largely unknown. Transition of microspores into the embryogenic state involves the degradation of earlier synthesized RNAs and proteins [80], [81], [82], particularly of gametophyte-specific proteins and metabolites (discussed in [30]). The isolation of a number of protease and ubiquitin-interacting genes from embryogenic microspores in independent studies supports this assumption [83], [84]. However, no key regulators involved in microspore reprogramming have been identified until now.

Our RNAi experiments showed that DCN1 is necessary for the stress-induced transition from microsporogenesis to microspore embryogenesis. Most microspores died during the stress treatment. Like in pollen development and zygotic embryogenesis, the mutant phenotype showed incomplete penetrance and variable expressivity which, in this case, allowed rescuing the surviving embryogenic microspores, enriching them by density
centrifugation, and culturing them to produce embryos. These embryos were arrested in their majority at the globular stage while a few continued with embryonic development and developed into plants.

As compared to freshly isolated microspores and unlike in normal pollen development, the DCN1 gene was slightly upregulated by the reprogramming stress treatment on the RNA and protein level. RNA and protein levels remained high in microspore-derived embryos until the globular stage, but were lower in late torpedo-shaped embryos. We conclude that DCN1 function is required until the globular stage of development, in line with the developmental arrest at the globular stage found in microspore-derived and zygotic embryos of Nt DCN1 RNAi lines.

The gain-of-function phenotype obtained by overexpressing DCN1 with the DC3 promoter indicated that DCN1 is not only necessary but also increases the potential for the phase change from gametophytic to sporophytic development of cultured microspores. Overexpression reduced the time of the stress treatment required to reprogram microspores. In cultured microspores that were not subjected to any stress treatment, the number of multicellular structures (early embryos) as well as the number of cells in these embryos were increased as compared to the wild type. However, these multicellular structures did not develop into advanced embryos. These experiments suggest that DCN1 may play a role in microspore reprogramming, similar to the genes that are required for the direct reprogramming of differentiated mammalian cells [85]. Possibly, a full priming of microspore totipotency would similarly require the action of multiple reprogramming genes.

This process of reprogramming towards totipotency seems to be different, furthermore, from regeneration from in vitro cultured somatic plant explants. There, the emergence of a root meristem-like, pluripotent cell mass termed callus, forming under the influence of hormones and followed by the formation of shoots and roots under different hormone conditions, does not seem to involve cellular reprogramming [86].

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Microscopy**

*Nicotiana tabacum* cv. “Petit Havana” SR1 plants were grown in at 25°C with a 16-h day. Microspores were isolated and cultured for maturation into pollen and for microspore embryogenesis according to [35].
Ovules were prepared according to [73]. After gently squeezing the fertilized ovules, the embryos were released and observed under a Zeiss Axioplan microscope equipped with Nomarski (DIC) optics. FDA staining and pollen germination assays were performed as described [35]. Pollen tube length was measured from images projected on a digitizer by using SIGMA-SCAN software V3.90 (Jandel Scientific, now http://www.systat.com, [87]). Histochemical GUS assays were performed according to [88].

Cloning

Full length cloning of Nt *DCNI* was carried out according to the instructions of the SMART RACE cDNA amplification kit (Clontech, http://www.clontech.com) using adapter and gene specific primers. RACE products were T/A-cloned (Invitrogen, http://www.invitrogen.com) and sequenced. The UNIVERSAL GENOME WALKER Kit (Clontech) was used for promoter isolation. The largest products were cloned into pCR2.1 and sequenced. The promoter region was inserted into the plant transformation vector pBI101.1. *DCNI* RNAi construct: A 270 bp fragment of the 3’ end of *DCNI* was cloned into pART69 [89] in antisense and sense orientation, separated by the *adh* Y5 intron and flanked by the 35S promoter and the *ocs* terminator, respectively. This cassette was cloned into pBI101.1 for plant transformation. ProSi (CaMV 35S promoter silencing) construct: A 1.4kb fragment of the 35S promoter from pART69 was cloned into the same vector in antisense orientation. This cassette was fused to a *Ubi* promoter fragment from pAHC25 and cloned into the pBIN:Hyg:TX vector (NCBI acc. no. Z37515). *DCNI* overexpression construct: Restriction sites were added to the 5’ and 3’ ends of Nt *DCNI* by PCR using specific primers. The product was T/A-cloned (Invitrogen), sequenced and cloned into pBI101.1 containing the DC3 promoter.

In Silico Sequence Analyses

Similarity searches were performed using BLAST [90] at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi applying default parameters and non-redundant databases. Promoter analyses were done at http://www.dna.affrc.go.jp/PLACE/ [91] and http://www-bimas.cit.nih.gov/molbio/proscan/, on-line analyses using the ExPASy pI/Mw tool [92]. Domain and motif analyses were performed with InterProScan [93] at http://www.ebi.ac.uk/interpro/, SMART at http://smart.embl-heidelberg.de/ [94], and at http://www.predictprotein.org/ [95].
Transformation of Tobacco and PCR Analysis of Transgenic Tobacco Plants

The constructs were transformed into *Agrobacterium* strain LBA4404 by electroporation. Tobacco leaf disks were transformed according to [96] and [97]. Transformants were selected on MS medium containing antibiotics, rooted and transferred to soil. DNA was isolated using the CTAB method [98]. The presence of the construct was verified by PCR, applying standard conditions.

In Situ and RNA Gel Blot Hybridization Analyses

In situ hybridization was done as described in [99]. Tissue sections were obtained using a microtome. Probes of a 5’-fragment (500 bp) of Nt *DCN1* were prepared using the DIG RNA Labelling Kit (Amersham Biosciences, now https://www2.gehealthcare.com). For RNA gel blot analyses, five µg of total RNA were loaded per lane. PCR-generated probes were labeled with [α-32P]dCTP using the RADPRIME DNA Labeling System (Invitrogen). RNA blots on Hybond-N membranes (Amersham Biosciences) were prepared and hybridized at 65°C according to standard procedures under high stringency. Hybridization was visualized on BIOMAX MR X-ray films (Kodak, http://www.kodak.com). For expression analysis by multiple tissue Northernns, a PCR-derived fragment of *DCN1*, corresponding to the original SSH-derived sequence was applied. A PCR-generated 697 bp 5’-fragment of *DCN1* was used for screening endogenous mRNA levels of RNAi plants, 431 bp of the Y5 intron of the pART69 vector served as a probe for screening the ectopic expression of the RNAi construct, *GAPDH* and 18S rRNA were used as internal controls for normalizing RNA loads.

Recombinant Proteins

A pUC:*DCN1* full length clone was used as a template for PCR and the product was cloned into pGEM-4T-1 (Promega, http://www.promega.com). After sequencing and transformation into *E. coli* ED3, expression and harvest of recombinant *DCN1* was done following standard procedures. For protein purification Glutathione Sepharose 4B (Amersham Biosciences) was used according to the manufacturer’s instructions. A rabbit antiserum was produced by Sanova Diagnostik (http://www.sanova.at). The At *CUL1*:His-tag vector was kindly provided by M. Estelle. Expression and purification was performed as recommended by the Ni-NTA purification system handbook (Invitrogen).
**Protein Gel Blot Analyses**

Proteins from 100 mg of plant tissue were isolated by standard protocols. The protein content was measured with a Bradford assay (BioRad, http://www.bio-rad.com). Ten to twenty µg of protein were separated in 10-12.5% SDS-PAGE. Equal protein load on nitrocellulose membrane was verified by Ponceau S staining after transfer or Coomassie-staining of the gel. Membranes were blocked in PBS-T with 5% milkpowder and incubated with Protein A purified polyclonal rabbit antiserum against recombinant Nt DCN1 (1:2500). AP-labeled anti-rabbit IgG (1:5000, Sigma-Aldrich, http://www.sigma.com) and CDPStar reagent (Amersham Biosciences) were used for photodetection on HYPERFILM ECL (Amersham Biosciences).

**Ubiquitin/NEDD8 Binding Assay and Pull-Down**

The binding assay was performed with ubiquitin-agarose (Sigma-Aldrich) and NEDD8-agarose (Boston Biochem. http://www.bostonbiochem.com), essentially as described in [26]. Initial binding was performed with 120µg recombinant Nt DCN1 and 5 µl (50 µg) of agarose-immobilized ubiquitin (7-15 µg/µl) or NEDD8 (5 µg/µl). Aliquots of starting reaction, washes and eluates were separated using 12.5% SDS-PAGE. Western detection was done as described above. For the pull-down, recombinant At CUL1:His protein (kindly provided by Mark Estelle) and recombinant Nt DCN1 were incubated with Ni-NTA beads (Qiagen, http://www1.qiagen.com). All washing and elution steps were performed according to user’s instructions.

**Accession Numbers**

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers DQ885939 (DCN1A), FJ976682 (DCN1B) and DQ885938 (promoter).

**ACKNOWLEDGMENTS**

We thank Kristina Belogradova and Svetlana Akimcheva for excellent management of transgenic lines, and Maria Kalyna for assistance with DIC and GUS assays. We are grateful to Ortrun Mittelsten Scheid and Barbara Hohn for commenting on the manuscript, to Marjori Matzke for helpful remarks, and Irina Sadovnik and Håvard Nyhagen Henriksen for critical reading. Fatima Touraeva and Maria Granilshikova are gratefully acknowledged for plant care. Mark Estelle kindly provided the AtCul1-His-tag construct. J.H. was supported by a DOC scholarship granted from the Austrian Academy of Sciences.
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BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-
regulates Arabidopsis anther dehiscence, pollen maturation, and filament elongation.
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TABLES

**Table 1.** Pollen viability and germination frequency in homozygous Nt *DCN1* knock-down lines.

<table>
<thead>
<tr>
<th>Line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Late unicellular microspores, viability&lt;sup&gt;b&lt;/sup&gt; %</th>
<th>Early bicellular pollen, viability&lt;sup&gt;b&lt;/sup&gt; %</th>
<th>Mature pollen, viability&lt;sup&gt;b&lt;/sup&gt; %</th>
<th>Pollen germination frequency&lt;sup&gt;c&lt;/sup&gt; %</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>90.2 ± 2.5</td>
<td>85.0 ± 2.9</td>
<td>80.3 ± 1.4</td>
<td>80.5 ± 2.1</td>
</tr>
<tr>
<td>RNAi L25</td>
<td>70.8 ± 1.5</td>
<td>61.2 ± 3.7</td>
<td>43.4 ± 1.3</td>
<td>27.2 ± 1.5</td>
</tr>
<tr>
<td>RNAi L35</td>
<td>68.3 ± 1.8</td>
<td>67.3 ± 3.1</td>
<td>32.2 ± 1.3</td>
<td>30.0 ± 1.8</td>
</tr>
<tr>
<td>RNAi L47</td>
<td>65.0 ± 1.4</td>
<td>40.3 ± 3.5</td>
<td>16.8 ± 1.6</td>
<td>9.0 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mature pollen was collected from wild-type and Nt *DCN1* RNAi lines L25, L35, and L47.

<sup>b</sup> Microspores were stained with FDA to evaluate viability.

<sup>c</sup> Pollen was cultured in germination medium GK, and germination frequency was scored under a light microscope by counting at least 300 pollen grains in three independent experiments.

**Table 2.** Pollen viability, pollen germination, and pollen tube growth in Nt *DCN1* overexpressor lines.

<table>
<thead>
<tr>
<th>Line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mature pollen, viability&lt;sup&gt;b&lt;/sup&gt; %</th>
<th>Pollen germination frequency&lt;sup&gt;c&lt;/sup&gt; %</th>
<th>Tube length 60 min (µm)</th>
<th>Tube length 120 min (µm)</th>
<th>Tube growth from 60 to 120 min (µm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>81.6 ± 3.2</td>
<td>77.3 ± 3.5</td>
<td>95 ± 18</td>
<td>358 ± 58</td>
<td>4.38</td>
</tr>
<tr>
<td>ox14</td>
<td>90.0 ± 3.5</td>
<td>82.8 ± 4.1</td>
<td>139 ± 34</td>
<td>752 ± 93</td>
<td>10.22</td>
</tr>
<tr>
<td>ox21</td>
<td>81.2 ± 2.5</td>
<td>61.3 ± 2.2</td>
<td>134 ± 26</td>
<td>611 ± 45</td>
<td>7.95</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mature pollen was collected from wild-type and overexpressor lines ox14 and ox21.

<sup>b</sup> Pollen was stained with FDA to evaluate viability.

<sup>c</sup> Pollen was cultured in germination medium GK and germination frequency was scored under the light microscope by counting at least 300 pollen grains in three independent experiments.
Table 3. Microspore embryogenesis with a two-day stress treatment in Nt DCNI overexpressor lines.

<table>
<thead>
<tr>
<th>Line a, duration of stress treatment for reprogramming</th>
<th>Embryogenic microspores %</th>
<th>Multi-cellular structures after two weeks %</th>
<th>Microspore-derived embryos after six weeks %</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt, 6-day stress treatment</td>
<td>82.5 ± 1.7</td>
<td>67.4 ± 0.9</td>
<td>45.4 ± 1.1</td>
</tr>
<tr>
<td>wt, 2-day stress treatment</td>
<td>41.6 ± 0.8</td>
<td>21.5 ± 1.5</td>
<td>11.5 ± 1.9</td>
</tr>
<tr>
<td>ox14, 2-day stress treatment</td>
<td>72.5 ± 1.5</td>
<td>57.0 ± 2.1</td>
<td>36.5 ± 1.4</td>
</tr>
<tr>
<td>ox21, 2-day stress treatment</td>
<td>69.6 ± 1.1</td>
<td>53.2 ± 1.5</td>
<td>33.1 ± 0.8</td>
</tr>
</tbody>
</table>

a Microspores at the late unicellular stage were isolated from wild-type and overexpressor lines and cultured for two days in starvation medium B at 33°C and then transferred to microspore embryogenesis medium AT3 at 25°C in the dark for six weeks until the formation of torpedo-shaped embryos.

Table 4. Microspore embryogenesis without stress treatment in Nt DCNI overexpressor lines.

<table>
<thead>
<tr>
<th>Lines a</th>
<th>Multi-cellular structures after ten days (%)</th>
<th>Average number of nuclei in multi-cellular structures after ten days b</th>
<th>Average number of nuclei after 28 days b</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.9 ± 0.3</td>
<td>2-3</td>
<td>2-12</td>
</tr>
<tr>
<td>ox14</td>
<td>10.6 ± 0.6</td>
<td>4-6</td>
<td>3-20</td>
</tr>
<tr>
<td>ox21</td>
<td>11.1 ± 0.6</td>
<td>3-6</td>
<td>2-18</td>
</tr>
</tbody>
</table>

a Microspores at the late unicellular stage were isolated from wild-type and overexpressor lines, respectively, and cultured in microspore embryogenesis medium AT3 at 25°C in the dark.

b Microspores were stained with DAPI after ten and 28 days of culture, and the frequency of multicellular structures and the number of nuclei these contained cells were estimated under the fluorescent microscope in 300 cells in three independent experiments.
**FIGURES AND LEGENDS**

**Figure 1.** Nt DCN1 Is a Highly Conserved 30kDa DCN1 Ortholog that Binds Ubiquitin and RUB/NEDD8 and Associates with Cullins.

(a) Sequence alignment of the two DCN1 paralogs (DCN1A and DCN1B) with DCN1 orthologs from different organisms, secondary structure prediction, and domain structure. Conserved amino acids are highlighted in grey, substitutions are boxed. Regions with a high probability of α-helix formation (H), as well as UBA, PONY, and EF-hand domains are indicated (EF1, EF2, canonical EF-hands are helix-loop-helix structural domains with an affinity to calcium). (b) Nt DCN1 binds ubiquitin and RUB/NEDD8. Recombinant Nt DCN1 was incubated with beads conjugated to either ubiquitin (5 µg/µl, mix, Ubi) or RUB/NEDD8.
(7-15 µg/µl, Nedd), respectively. Aliquots of wash fractions (W1–W8) and eluates (elu) were subjected to PAGE, and Western blots were probed with anti-DCN1. (c) Pull-down of Nt DCN1 with At CUL1:His fusion protein bound to Ni-NTA beads. Aliquots of wash fractions (W1, W2) and eluates (1-3) were subjected to PAGE and Western blotting with anti-Nt DCN1. The band above 86 kDa in the first wash fraction (W1) appears to be an unspecific artifact. The association of recombinant Nt DCN1 with recombinant His:AtCul1 bound to the beads is disrupted upon elution (fractions 1 and 2). The reaction was carried out with recombinant proteins in absence of Nedd8. The lower panel represents a control assay without Cul1.

Figure 2. Nt DCNI is expressed throughout the plant body.

Expression pattern of Nt DCNIpro::GUS in seedling (a), lateral meristem (b), lateral root initial (c), apical meristem (d), first leaf primordium (e), unfertilized ovaries (f), early embryo and endosperm (g) (light microscopy, no GUS staining is visible due to weak expression), early embryo and endosperm (h) (dark-field image showing weak GUS expression as pink dots and strong signals in blue), mature seeds (j), imbibed seeds (k), early seedling (l).
Figure 3. Nt DCNI is expressed during pollen development and microspore embryogenesis. (a-c) Expression pattern of DCNI by Northern (a and b) and Western blotting (c) in vegetative tissues (a), during microspore embryogenesis (b) and pollen development (c). Northern blots were hybridized with the radiolabeled 3’ UTR of Nt DCNI. Lower panels show loading controls of 18S rRNA in (a and b), Coomassie staining in (c). infl., inflorescences, y, young leaf, m, intermediate leaf, o, old leaf, stem, stem, root, root, um, unicellular microspores, sm, stressed microspores, em, microspore-derived embryos (after 25 days and 50 days), mp, mature pollen, bp, bicellular pollen. (d) Expression pattern of DCNI detected by in situ hybridization. sm, stressed microspores, dm, dividing microspores, mc, multicellular structures, ge, globular embryos, ce, cotyledon stage embryos. (e) Nuclear status assayed by DAPI staining and Nt DCNIpro::GUS expression. um, unicellular microspores, mp, mature pollen, sm, stressed microspores, lg, late globules.
Figure 4. Nt DCN1 is required for microspore and zygotic embryogenesis as well as for pollen development.

(a) Northern and Western blots of three homozygous DCN1 RNAi lines and their respective, re-transformed promoter-silencing lines compared to the wild-type. Endogenous Nt DCN1 RNA levels were detected with a DCN1-derived probe, comprising the coding region, and double-stranded hairpin RNA levels by probing against the intron of the RNAi construct. GAPDH was used as a loading control. hp, hair-pin probe, PS, promoter silencing lines. (b) Pollen formation from cultured microspores in RNAi and promoter silencing lines. wt, wild-type, PS, promoter silencing lines, um, unicellular microspores, mp, mature pollen, gp, germinated pollen. Bars: 30 µm. (c) DIC microscopy pictures of embryo development and seed formation in RNAi line L47 compared to the wild-type. Early development of zygotes in
both RNAi and wild-type plants was normal and synchronous, but embryos in RNAi lines were arrested around the globular stage, or showed malformed heart and cotyledon stages. z, zygote, ge, globular embryo, he, heart stage embryo, te, torpedo stage embryo. Bars: z = 20 µm, ge, he, te = 200 µm. (d) Seed pods were smaller in RNAi lines, here shown for L47, immature seeds developed asynchronously, and only a few mature seeds developed in contrast to the wild-type. is, immature seeds, ms, mature seeds. (e) Microspore embryogenesis in wild-type, RNAi (L47) and promoter silencing line PS-L47 (PS). um, unicellular microspores, sm, stressed microspores, ge, globular embryos, ce, cotyledon stage. Bars: um, sm = 30 µm, ge = 100 µm, ce = 2 mm.

Figure 5. Overexpression of Nt DCNI accelerates pollen tube growth and promotes reprogramming of microspores in vitro.
(a) Overexpression of Nt DCN1 in microspores determined by Western blotting. ctrl, recombinant Nt DCN1, wt, wild-type, ox14, overexpressor line 14, ox21, overexpressor line 21. In the lower panel the Coomassie-stained gel is shown as a loading control. (b) RT-PCR showing the time-course of DCN1 expression during the six-day stress treatment leading to the reprogramming of microspores. DCN1 expression is elevated in unstressed and stressed microspores of overexpressor lines ox14 and ox21 compared to the wild-type. u, unstressed microspores, 2, 2-day-stressed microspores, 4, 4-day-stressed microspores, 6, 6-day-stressed microspores. (c) In overexpressor lines two days of stress treatment were sufficient to induce embryogenesis at a high frequency compared to the wild-type. Pictures of microspore embryogenesis in ox14 were taken after two weeks (globular stage) and six weeks (cotyledon stage). Bars: 300 µm. (d) Pollen tube growth in wild-type and overexpressor lines. Pictures were taken after 30 and 120 minutes, respectively. wt, wild-type, ox14, overexpressor line 14, ox21, overexpressor line 21. Bars: 30 µm.

SUPPORTING INFORMATION

Supporting Information Fig. 1. Pull-down of Recombinant Nt DCN1 with At CUL1:His Bound to Ni-NTA Beads. Aliquots of wash fractions (W1, W2) and eluates (1-4) were subjected to PAGE and Coomassie staining. The band at 86 kDa in fractions 1 to 3 corresponds to co-eluting His:AtCul1 while DCN1 manifests above 26 kDa.
Supporting Information Fig. 2. Expression of Nt DCN1 in Microspores of Nt DCN1 RNAi Plants. Western blot with protein isolated from wt and RNAi line 25, respectively; LU late unicellular microspores, str stressed microspores, gl globular embryos, T/C torpedo/cotyledon stage embryos.

Supporting Information Fig. 3. Hyper-Methylation of the 35S Promoter during Transcriptional Silencing of the Nt DCN1 RNAi Construct in “Knock-Up” Double Transformants. (a) Schemes of the 35S promoter region of RNAi and promoter silencing constructs. Black bars indicate the localisation of the probe used for Southern blotting; M/H indicates restriction sites for the methylation-sensitive enzyme HpaII (the isoschizomer of methylation-insensitive MspI); the schemes are not drawn to scale. (b) Southern blot performed with HpaII-digested DNA of Nt DCN1 RNAi lines 35 and 47 and derived retransformed, promoter-silenced lines (35PS1, 47PS1, 47PS2, 47PS3); plasmids of the
RNAi construct (RNAi) and the 35S TGS (ProSi) construct with partially digested DNA, respectively, are shown as size control.

Supporting Information Table 1. In Vitro Maturation of Microspores Isolated from Homozygous Nt DCNI RNAi Plants. Microspores were isolated from anthers of wild-type and RNAi plants and were cultured in medium AT3 [35]. After 5 d, pollen grains were spun down and cultured in medium P for 1 d.

<table>
<thead>
<tr>
<th>Line</th>
<th>Late unicellular microspores, viability %</th>
<th>In vitro matured pollen grains, b %</th>
<th>Pollen germination frequency c %</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>92.1 ± 2.9</td>
<td>78.3 ± 2.9</td>
<td>74.5 ± 3.2</td>
</tr>
<tr>
<td>RNAi L25</td>
<td>68.8 ± 2.1</td>
<td>40.4 ± 1.9</td>
<td>25.1 ± 1.8</td>
</tr>
<tr>
<td>RNAi L35</td>
<td>65.3 ± 2.6</td>
<td>31.9 ± 2.1</td>
<td>27.7 ± 2.0</td>
</tr>
<tr>
<td>RNAi L47</td>
<td>63.0 ± 2.2</td>
<td>14.5 ± 1.9</td>
<td>9.3 ± 0.5</td>
</tr>
</tbody>
</table>

a As seen under an inverted microscope, live microspores in cultures were turgescent while dead microspores were shriveled.

b In vitro matured pollen grains were enlarged and had a triangular or rectangular shape.

c In vitro matured pollen was cultured in germination medium PEG8000, and germination frequency was scored under a light microscope by counting at least 300 pollen grains in three independent experiments.