Post-translational modifications regulate the activity of the growth restricting protease DA1

Ying Chen^{1,2}, Dirk Inzé^{1,2,*} and Hannes Vanhaeren^{1,2,*}

¹Department of Plant Biotechnology and Bioinformatics, Ghent University, B-9052 Ghent, Belgium

²Center for Plant Systems Biology, VIB, B-9052 Ghent, Belgium

*Shared senior authors.

[†]Author for contact: dirk.inze@psb.vib-ugent.be

Corresponding author:

Dirk Inzé

VIB Center for Plant Systems Biology

Ghent University, Department of Plant Biotechnology

Technologiepark 71

B-9052 Ghent (Belgium)

Tel.: +32 9 3313800; Fax: +32 9 3313809; E-mail: dirk.inze@psb.vib-ugent.be

ORCID IDs: 0000-0003-1182-0875 (Y.C.); 0000-0002-3217-8407 (D.I.); 0000-0003-3343-377X (H.V.)

Highlight

The activity of the protease DA1 is regulated by complex crosstalk of post-translational modifications, such as (de)ubiquitination and phosphorylation, and determines the stability of positive growth regulators.

Keywords: cell proliferation, DA1, degradation, development, N-degron pathway, organ size, phosphorylation, protease, proteostasis, ubiquitination.

Running title: Organ size control by the DA1 protease

Abstract

Because plants are a primary food source and can form the basis for renewable energy resources, the final size of their organs is by far the most important trait to tackle when seeking increased plant productivity. Being multicellular organisms, plant organ size is mainly determined by the coordination between cell proliferation and cell expansion. The protease DA1 limits the duration of cell proliferation and hereby restricts final organ size. Since its initial identification as a negative regulator of organ growth, various transcriptional regulators of DA1, but also interacting proteins, have been identified. These interactors include cleavage substrates of DA1, but also proteins that modulate the activity of DA1 through post-translational modifications, such as

ubiquitination, deubiquitination and phosphorylation. In addition, many players in the DA1 pathway display conserved phenotypes in other dicot and even monocot species. In this review, we give a timely overview of the complex, but intriguing molecular mechanisms that fine-tune the activity of DA1 and therefore final organ size, and we lay out a roadmap to identify and characterize substrates of proteases and frame the substrate cleavage events in their biological context.

Introduction

Proteases degrade non-functional proteins for amino acid recycling by cleaving N- or C-terminal peptide bonds and can be grouped into six categories based on their nucleophilic amino acid at their active site: aspartic, cysteine, serine, threonine, glutamic and asparagine proteases; metalloproteases deprotonate a water molecule, leading to a nucleophilic attack on a carbonyl peptide bond of a substrate (Oda, 2012). The *MEROPS* peptidase database (<http://merops.sanger.ac.uk>), contains over 900 proteases encoded by the Arabidopsis genome (Rawlings *et al.*, 2018). Their distribution and family size are remarkably conserved within the plant kingdom (van der Hoorn, 2008). Besides their ancestral housekeeping functions, proteases are found to be key regulators in different growth and developmental processes, including embryo development (Johnson *et al.*, 2005), seed germination (Martinez *et al.*, 2019), flowering time (Murtas *et al.*, 2003), shoot and root growth (Yang *et al.*, 2018; Soares *et al.*, 2019) and senescence (Buet *et al.*, 2019). Proteases also participate in programmed cell death (PCD), for example during the formation of xylem, such as XYLEM CYSTEINE PROTEASE 1 (XCP1), XCP2 and METACASPASE 9 (MC9) (Avci *et al.*, 2008; Bollhöner *et al.*, 2013). Proteases also regulate plant responses to biotic and abiotic stimuli. Hypersensitive responses (HRs) in plants are caused by pathogenic attacks, followed by a rapidly induced cell death at the infected sites. The VACUOLAR PROCESSING ENZYME (VPE) is for example an essential protease involved in PCD during a virus-induced HR (Hatsugai *et al.*, 2004). An atypical aspartic protease, ASPARTIC PROTEASE IN GUARD CELL1 (ASPG1), can positively regulate abscisic acid sensitivity in guard cells during drought stress, and ectopic expression of *ASPG1* improves drought tolerance (Yao *et al.*, 2012).

From the small leaves of duckweed to giant Banana tree leaves, organ size is one of the most obvious differences between plant species. The final size of plant organs is strictly controlled by intrinsic developmental signals and environmental factors (Gonzalez *et al.*, 2012; Czesnick and Lenhard, 2015). The embryo and endosperm originate from the double fertilization process, while the seed coat is typically of maternal origin (Li and Li, 2014). Therefore, seed size is regulated by the coordinated growth of maternal sporophytic and zygotic tissues (Li and Li, 2014). Regardless of the difference in origin, organ size is basically determined by the number of cells they contain and the extent to which these cells expand (Johnson and Lenhard, 2011). The total cell number and size are in their turn influenced by the duration of cell proliferation and the extent of cell expansion, respectively (Gonzalez *et al.*, 2012). Leaf growth is mainly achieved by cell proliferation during its early

developmental stages. At this stage, new cells are generated that have a relatively constant and small size (Gonzalez *et al.*, 2012). During the transition to cell expansion, cells gradually exit the mitotic cell cycle and start to expand and differentiate (Donnelly *et al.*, 1999). The division of Arabidopsis root cells is mainly maintained by the stem cell niche, which consists of the mitotically inactive quiescent center (QC) cells and the surrounding mitotically active stem cells (Scheres, 2007; Dinneny and Benfey, 2008). These latter stem cells generate transit-amplifying cells, which undergo additional divisions in the proximal meristem and then differentiate in the meristem transition zone, prior to rapidly expanding in length in the elongation zone (Ubeda-Tomas and Bennett, 2010). During the generative stage, the Arabidopsis shoot meristem gives rise to floral meristems, which initiate floral organs. Seed size is controlled by the interaction between endosperm growth and integument proliferation and elongation. The early stage of seed development is characterized by the active proliferation and growth of the endosperm, which largely determines the final seed size (Boisnard-Lorig *et al.*, 2001). In contrast, growth of the embryo occurs primarily during the later phase at the expense of the endosperm (Jurgens and Mayer, 1994).

Cell proliferation and expansion are regulated at multiple molecular levels. Several transcriptional regulators, such as GROWTH REGULATING FACTORS (GRFs) (Kim and Tsukaya, 2015), ANGUSTIFOLIA3 (AN3) (Ercoli *et al.*, 2018; Liu *et al.*, 2019; Zhang *et al.*, 2019a), AINTEGUMENTA (ANT) (Elliott *et al.*, 1996; Mizukami and Fischer, 2000; Ohto *et al.*, 2005) and PEAPOD (PPD) (White, 2006; Gonzalez *et al.*, 2015) determine the size of leaves, roots, flowers and seeds by regulating cell proliferation. MicroRNAs (miRNAs) are a class of short non-coding RNAs, which regulate gene expression in plants and animals (Jones-Rhoades *et al.*, 2006). *miR319*, the first plant miRNA discovered, regulates the development of plant organs by targeting different genes, including *TEOSINTE BRANCHED1*, *CYCLOIDEA*, and *PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTORS* (TCPs) (Koyama *et al.*, 2017). Overexpression of *miR319* (*JAGGED AND WAVY* (*JAW*)) results in an increased cell proliferation at the leaf edges, leading to larger leaves with a wavy curvature (Palatnik *et al.*, 2003). *miR396* negatively regulates cell proliferation by targeting most, but not all *GRFs* (Rodriguez *et al.*, 2010; Rodriguez *et al.*, 2015).

Also phytohormones play an important role in organ growth. Overexpression of *GA20-oxidase 1* (*GA20ox1*), encoding a rate-limiting enzyme in the gibberellins (GAs) biosynthesis pathway, increases organ size by promoting cell division and expansion (Huang *et al.*, 1998; Gonzalez *et al.*, 2010; Nam *et al.*, 2017). GAs trigger the rapid degradation of the growth-repressing DELLA proteins, and in quadruple-DELLA mutants, the rate of cell division rate is higher (Achard *et al.*, 2009). Brassinosteroids (BRs) were discovered as hormones that stimulate both plant cell elongation and division. BR signaling is directed by BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE1 (BAK1) (Rusinova *et al.*, 2004); overexpression of *BRI1* under the control of its own promoter extends the period of cell proliferation, leading to enlarged leaf organs (Wang *et al.*, 2001; Gonzalez *et al.*, 2010). Members of the *SMALL AUXIN UP RNA* (*SAUR*)

gene family are rapidly and strongly induced by auxin (Hagen and Guilfoyle, 2002); overexpression of *GFP-SAUR19* increases leaf size by promoting cell expansion (Spartz *et al.*, 2012), whereas *SAUR36* negatively regulates cell size (Hou *et al.*, 2013).

Ubiquitination also plays a prominent role in organ size control as a versatile post-translational modification. Ubiquitination is catalyzed by the sequential action of three enzymes: the ubiquitin-activating enzyme (E1), which activates ubiquitin molecules; the ubiquitin-conjugating enzyme (E2), which accepts the activated ubiquitin from E1, forming an E2-ubiquitin intermediate; and the ubiquitin ligase (E3), which facilitates the transfer of ubiquitin from the E2-ubiquitin intermediate to the substrate protein (Callis, 2014). Two RING E3 ligases, BIG BROTHER/ENHANCER 1 OF DA1 (BB/EOD1, hereafter referred to as BB) and DA2, negatively regulate cell proliferation and hence organ size (Disch *et al.*, 2006; Xia *et al.*, 2013). In addition, the ANAPHASE-PROMOTING COMPLEX/CYCLOSOME (APC) subunit APC10 positively regulates the cell division rate during the early stages of leaf development (Eloy *et al.*, 2011), and the F-box protein STERILE APETALA (SAP) promotes meristemoid proliferation by controlling the stability of KIX and PPD proteins (Wang *et al.*, 2016; Li *et al.*, 2018).

In this review, we will focus on the ubiquitin-dependent protease DA1, which stands for “large” in Chinese, a negative regulator of organ size in *Arabidopsis thaliana* and many other plant species. We will discuss in depth the latest findings on the mode of action of this protease and its role in defining plant organ size. In addition, we will lay out a roadmap to identify and characterize substrates of proteases and frame the substrate cleavage events in their biological context.

DA1 regulates organ size by restricting cell proliferation

DA1 was originally identified in a genetic screen for larger organs (Li *et al.*, 2008). The *da1-1* mutant, which harbors a point mutation leading to an amino acid change from arginine to lysine at position 358 (DA1^{R358K}), produces larger leaves, flowers, siliques and seeds (Fig. 1), due to an extended period of cell proliferation (Li *et al.*, 2008; Vanhaeren *et al.*, 2017; Dong *et al.*, 2020). Seven DA1-related (DAR) genes are encoded by the *Arabidopsis* genome, of which *DAR1* and *DAR2* are the family members most closely related to *DA1*. Next to amino acid similarities, *DA1*, *DAR1* and *DAR2* also have a similar expression pattern, which is the strongest in young, developing leaves (Li *et al.*, 2008; Peng *et al.*, 2015). Single knockouts of *DA1*, *DAR1* and *DAR2* only display subtle phenotypes (Li *et al.*, 2008; Peng *et al.*, 2015; Dong *et al.*, 2017), whereas the double mutant of *DA1* and *DAR1* (*da1-ko1_dar1-1*) shows an obvious increase in organ growth, similar to *da1-1* plants (Li *et al.*, 2008). In addition, constitutive ectopic expression of DA1^{R358K} also mimics the *da1-1* mutation (Fig. 1), suggesting that this mutant protein exerts a dominant-negative function towards DA1 and DA1-related proteins (Li *et al.*, 2008). Interestingly, the growth-enhancing effect of the interfering *da1-1* allele (DA1^{R358K}) can be suppressed by a nearby additional mutation in the coding sequence of DA1 (Fig. 2, *sod1-1*, DA1^{R358K,L362F}) (Li *et al.*, 2008). Likewise,

a premature stop codon (*sod2-1*, DA1^{R358K,Q435}) that interrupts the HEMMHAX₁₅EE peptidase sequence (Fig. 2) also abolishes the *da1-1* phenotype. Most probably, both additional mutations render the interfering *da1-1* allele completely dysfunctional. In contrast, *35S::GFP-DA1* overexpressing plants produce smaller organs with fewer cells (Vanhaeren *et al.*, 2017) (Fig. 1). DA1 also controls seed size maternally. Seeds produced by a *da1-1* plant as pollen acceptor, regardless of the genotype of the pollen donor, are consistently heavier and larger than those produced by maternal wild-type (WT) plants, and *da1-1* mutant pollen applied to a WT plant produces WT-sized seeds (Li *et al.*, 2008). In addition, DA1 limits the initiation of axillary meristems. In long-day growth conditions, *da1-1* plants produce fewer branches and axillary buds as compared to WT plants (Li *et al.*, 2020) (Fig. 1). In contrast with *da1-1* and the *da1-ko1_dar1-1* double mutant, the *da1-ko1_dar1-1_dar2-2* triple mutant produces dwarfed rosette leaves containing small epidermal and palisade cells with lower ploidy levels (Peng *et al.*, 2015). Remarkably, this triple mutant exhibits larger flowers and seeds, which illustrates the tissue-dependent effect of these proteins (Peng *et al.*, 2015). Overexpression of either *DA1*, *DAR1* or *DAR2* in the *da1-ko1_dar1-1_dar2-2* triple mutant restores its phenotype back to WT (Peng *et al.*, 2015).

The growth-regulating function of DA1 is conserved throughout different Arabidopsis natural accessions, as well as other species (Fig. 1). For example, overexpression of DA1^{R358K} in 17 Arabidopsis natural accessions causes to a different extent the formation of larger mature leaves, resulting from an increased cell number (Vanhaeren *et al.*, 2017). Overexpression of Arabidopsis DA1^{R358K} in *Brassica napus* also leads to larger organs, including seeds, cotyledons, leaves, flowers and siliques (Wang *et al.*, 2017). In the maize inbred line DH4866, overexpression of a similar dominant-negative mutation of *ZmDA1* or *ZmDAR1* results in smaller but more epidermal cells in developing leaves and improves grain yield through an increased grain weight and number, whereas overexpression of the WT genes reduces seed size (Xie *et al.*, 2018). In addition, an RNAi-mediated downregulation of *TaDA1*, the wheat DA1 homolog, leads to an increased kernel size and weight, whereas its overexpression results in the opposite effect (Liu *et al.*, 2020a). In soybean, the ectopic expression of WT *GsoDA1* had no effect on seed size, but seed germination was enhanced under salinity (Zhao *et al.*, 2015). The downregulation or ectopic expression of mutated DA1 genes in soybean, which appears to be the most successful strategy, remains however unexplored.

Protein domains of DA1 and their function in controlling its activity

DA1 is a latent protease which is catalytically activated upon monoubiquitination of four consistent lysine residues at its C-terminal domain, K381, K391, K474 and K519 (Fig. 2) (Dong *et al.*, 2017). Mutating these four lysines to arginine results in the ubiquitination of alternative lysine residues (K95, K221, K484, K514) (Fig. 2), leading to an equal catalytic activity of DA1, demonstrating that there is a preference but no specificity for these lysine residues (Dong *et al.*, 2017). DA1 contains two ubiquitin-interacting motifs (UIMs) (Hicke *et al.*, 2005), a

single zinc-binding LIM domain and a conserved C-terminus that harbors a peptidase motif (Dong *et al.*, 2017). The two UIMs are located N-terminally and are essential for the ubiquitination of DA1 (Fig. 2). *In vitro*, both DA1 UIMs can bind ubiquitin. Mutations in UIM2, but not in UIM1, decrease the ubiquitination of DA1 and lead to a failure in complementing the large petal size in the double mutant *da1-ko1_dar1-1* (Dong *et al.*, 2017), which highlights its crucial role in the regulation of DA1 activity.

LIM domains are characterized by a canonical Lin-11, Isl-1 and Mec-3 domain (Freyd *et al.*, 1990; Hiyama *et al.*, 1999), contain two independent zinc fingers with the consensus amino acid sequence of CX₂CX₁₆₋₂₃HX₂CX₂CX₂CX₁₆₋₂₁CX₂₋₃(C/H/D) (Kadmas and Beckerle, 2004) and have been shown to play a role in protein-protein interactions (Zheng and Zhao, 2007). In plants, LIM domain proteins are classified into two categories, based on the number of LIM domains: single-domain LIM proteins, such as DA1, DAR1 and DAR2, and double-domain LIM proteins (Srivastava and Verma, 2017). By mutating the LIM domain in DA1 (DA1^{C247Y}), its ubiquitination is abolished and the protease cannot be activated (Dong *et al.*, 2017).

The conserved C-terminal region of DA1 contains an extended sequence motif, HEMMHX₁₅EE, which is a zinc aminopeptidase active site found in the clan MA endopeptidases (van der Hoorn, 2008). By mutating the HEMMH motif to AEMMA (DA1^{H418A,H422A}), DA1 can still be ubiquitinated, but its peptidase activity is abolished (Fig. 2). Accordingly, DA1^{H418A,H422A} fails to complement the large *da1-ko1_dar1-1* phenotype (Dong *et al.*, 2017). In addition, the DA1^{R358K} mutation, situated in a conserved region at the C-terminus, does not influence the ubiquitination of DA1, but its peptidase activity is significantly reduced (Dong *et al.*, 2017), implying the *da1-1* phenotype may result from a decreased peptidase activity.

Over the last years, multiple regulators, interactors and substrates of DA1 have been identified through genetic and proteomic screens (Fig. 3A). First, we will discuss the current knowledge of the transcriptional control of DA1, followed by the regulation of its catalytic activity. Finally, we will give an overview of the currently known substrates and their role in controlling organ size.

Regulation of *DA1* transcription

Recent studies have demonstrated that the expression of *DA1* is regulated by transcription factors and chromatin-remodeling enzymes affecting the epigenetic histon code.

Histone H2B monoubiquitination is one type of histone modification, which affects histone methylation and influences the expression level of the nearby genes (Pavri *et al.*, 2006; Shilatifard, 2006). Evidence suggests that also DA1 is subject to this type of regulation. The OTUBAIN-LIKE CYSTEINE PROTEASES (OTU) family can remove the monoubiquitin marks from histones (Fig. 3A) (Keren and Citovsky, 2016). *OTU1* is expressed in both the cytoplasm and nucleus and loss of function of *OTU1* results in smaller organs such as seeds, rosettes and stems, mainly caused by a decreased cell proliferation (Keren *et al.*, 2020). In *OTU1* T-DNA insertion mutants,

the *DA1* chromatin regions have higher levels of H2B monoubiquitination and the expression of *DA1* is increased, suggesting that OTU1 negatively regulates the expression of *DA1* (Keren *et al.*, 2020). Also the expression of a regulator of *DA1* activity, *DA2* (see further), is similarly affected in *otu1*.

Next to chromatin remodeling, the expression of *DA1* is regulated by CUP-SHAPED COTYLEDON 2 (*CUC2*) and *CUC3*. *CUC2* was the first identified NAC (NAM/ATAF1,2/*CUC2*)-encoding gene of Arabidopsis, and plays a role in shoot meristem initiation (Vroemen *et al.*, 2003; Hibara *et al.*, 2006). Both *CUC2* and *CUC3* are expressed in developing leaves (Hibara *et al.*, 2006); *CUC2* and *CUC3* directly bind to the promoter of *DA1* in the core sequence CGT[G/A] site and positively regulate its expression, increasing the initiation of axillary meristems (Li *et al.*, 2020).

Regulation of the activity of *DA1*

The RING E3 ligases *BB* and *DA2* interact both genetically and physically with *DA1* (Li *et al.*, 2008; Xia *et al.*, 2013), but the double mutant *bb_da2-1* has an additive effect compared to its parental lines, suggesting these E3 ligases work independently from each other (Xia *et al.*, 2013) (Fig. 3A-B).

BB is mainly expressed in proliferating tissues of leaves, flowers and in the vasculature, but its expression rapidly decreases during cell differentiation (Disch *et al.*, 2006), which is similar to the expression pattern of *DA1* (Peng *et al.*, 2015). Mutations in *BB* lead to an increased organ size, caused by an increased duration of cell proliferation (Disch *et al.*, 2006). In contrast, higher levels of *BB* reduce growth and accelerate senescence (Disch *et al.*, 2006; Vanhaeren *et al.*, 2017). The RING domain is essential for the catalytic activity of E3 ubiquitin ligases, as it binds to an E2~ubiquitin thioester and activates the discharge of its ubiquitin cargo (Deshaies and Joazeiro, 2009). Mutations in the RING domain of *BB* (*BB*^{C197S_C200S}) hence lead to catalytic deficiency (Disch *et al.*, 2006). Mutations in *BB* greatly enhance the *da1-1* phenotype (Li *et al.*, 2008) and the double mutant *da1-1_bb* displays synergistic effects on the size of seeds, flowers and leaves through a substantial increase in cell number (Li *et al.*, 2008; Dong *et al.*, 2017; Vanhaeren *et al.*, 2017). In addition, the double mutant *da1-1_bb* is delayed in its development and exhibits a longer life span (Li *et al.*, 2008; Vanhaeren *et al.*, 2017). *BB* can monoubiquitinate multiple lysine residues in *DA1*, *DAR1* and *DAR2*, leading to their catalytic activation (Fig. 3A-B) (Dong *et al.*, 2017).

DA2 is also expressed in young tissues, such as developing roots, cotyledons, leaves and inflorescences (Xia *et al.*, 2013). Inactivation of *DA2* produces larger organs such as seeds, flowers and leaves, whereas overexpression of *DA2* results in the opposite effect (Xia *et al.*, 2013). *DA2* and *DA1* act synergistically and the double mutant *da1-1_da2-1* generates larger seeds and petals than the WT and the single mutants (Xia *et al.*, 2013). Similarly as *DA1*, *DA2* functions maternally to regulate seed size by affecting cell proliferation in the maternal integuments, in concert with *DA1* (Xia *et al.*, 2013). *DA1* physically interacts with *DA2* through its

conserved C-terminal region (Xia *et al.*, 2013). Mutagenizing the RING domain in DA2 (DA2^{C59S}) leads to an abolished E3 ligase activity (Xia *et al.*, 2013). As BB, DA2 can interact and monoubiquitinate DA1, DAR1 and DAR2 at several lysine residues, leading to the catalytic activation of these proteases (Fig. 3A-B), but DA2 appears to work with a higher efficiency than BB (Dong *et al.*, 2017).

The antagonists of E3 ligases are deubiquitinating enzymes (DUBs) (Isono and Nagel, 2014), from which the ubiquitin-specific proteases (UBPs) are the largest family. In *Arabidopsis*, 27 UBPs are characterized by their conserved UBP domain (Yan *et al.*, 2000), that contains two short but conserved motifs, known as cysteine and histidine boxes (Amerik and Hochstrasser, 2004). These two motifs include a triad of catalytic residues, which are critical for the catalytic activity of the DUB (Amerik and Hochstrasser, 2004). UBP12 and UBP13 contain a unique meprin and TRAF homology (MATH) domain, and co-localize and associate with DA1, DAR1 and DAR2 (Vanhaeren *et al.*, 2020). More specifically, they can deubiquitinate and hereby deactivate DA1, DAR1 and DAR2 (Fig. 3A-B). Ectopic expression of *UBP12* or *UBP13* and, hence, low levels of activated DA1 proteins, result in a strong reduction of leaf size and epidermal cell area, and a decrease in endoreduplication (Vanhaeren *et al.*, 2020), similar to *da1-ko1_dar1-1_dar2-1* mutants (Peng *et al.*, 2015). In *ubp12-2* plants, which have lower transcript levels of both *UBP12* and *UBP13* (Cui *et al.*, 2013) and therefore might have higher levels of ubiquitinated DA1, DAR1 and DAR2, the number of leaf cells and, hence, final leaf area are reduced (Vanhaeren *et al.*, 2020). Similar phenotypes are observed in *GFP-DA1* overexpressing plants (Vanhaeren *et al.*, 2017) and mutants of *UBP15*, which encodes a cleavage substrate of DA1 (see further) (Liu *et al.*, 2008; Du *et al.*, 2014).

In addition, the peptidase activity of DA1 can also be regulated by phosphorylation (Dong *et al.*, 2020). The brassinosteroid receptor BRI1 and its co-receptor BAK1 directly bind and phosphorylate DA1, hereby reducing its peptidase activity (Dong *et al.*, 2020) (Fig. 3B). Expressing phospho-mimics of DA1 (Fig. 2, DA1^{S363D,T367D,T370D}) in *da1-ko1_dar1-2* only partially complements the enlarged phenotype, whereas expressing a non-phosphorylatable DA1 (DA1^{S363A,T367SA,T370A}) in *da1-ko1_dar1-2* results in a stronger growth reduction, indicating that phosphorylation reduces DA1's growth-repressive activity (Dong *et al.*, 2020). Furthermore, a combination of higher BRI1 levels with a reduced DA1 peptidase activity in *BRI1^{OE}_da1-1* plants leads to a synergistic effect in leaf growth (Vanhaeren *et al.*, 2014). In addition, phosphorylation of DA1 does not influence its ubiquitination (Dong *et al.*, 2020), indicating that phosphorylation works independently from ubiquitination in regulating the peptidase activity of DA1. Interestingly, in contrast to unphosphorylated DA1 and DA1^{S363A,T367SA,T370A}, phospho-mimicking DA1 proteins (DA1^{S363D,T367D,T370D}) are able to homodimerize, likely through their LIM or LIM-like domain, and high levels of brassinolide (BL) triggers the formation of high-molecular-weight (HMW) forms of DA1 (Dong *et al.*, 2020). Depending on the protein, dimerization can either have a positive or negative effect on their enzymatic activity (Marianayagam *et al.*, 2004). High levels of BRs, and by consequence an increased phosphorylation of DA1, can result in HMW complexes that are enzymatically less active (Dong *et al.*, 2020). This, together with the observation that BRASSINAZOLE-RESISTANT 1 (BZR1), a positive transcriptional regulator

of the BR signaling pathway, represses the transcription of *CUC2* and *CUC3* (Gendron *et al.*, 2012), which positively regulate *DA1* transcript levels (Li *et al.*, 2020), demonstrates that BR signaling is highly intermixed in the *DA1* pathway.

Several studies have elucidated that the peptidase activity of *DA1* is tightly controlled at multiple levels. The level of active, ubiquitinated *DA1* is regulated by two E3 ligases (Dong *et al.*, 2017), two deubiquitination enzymes (Vanhaeren *et al.*, 2020), through degradation of the activating E3 ligases by *DA1* itself (Dong *et al.*, 2017) and upon *BRI1/BAK1*-mediated phosphorylation (Dong *et al.*, 2020). These three independent routes to limit the catalytic activity of *DA1* emphasize the importance to tightly regulate this protease in a balanced manner to achieve a normal development of plant organs. The complex molecular networks that regulate the activity of *DA1* are likely coordinated by developmental and/or environmental signals, but remain entirely elusive until now.

***DA1* cleavage substrates**

After proteolytic cleavage, the neo-N-terminus can expose a stabilizing or destabilizing amino acid (Dissmeyer, 2019). In the latter case, the E3 ligase PROTEOLYSIS 6 (PRT6) can specifically recognize positively charged or Type 1 residues (Garzón *et al.*, 2007) and aromatic hydrophobic or Type 2 residues can be recognized by the E3 ligase PRT1 (Potuschak *et al.*, 1998). Subsequently, these protein fragments are polyubiquitinated and rapidly degraded by the proteasome (Gibbs *et al.*, 2016). Upon ubiquitination by BB or *DA2*, *DA1* is catalytically activated and can cleave downstream targets. Remarkably, *DA1* also cleaves these E3 ligases in a negative feedback loop (Dong *et al.*, 2017) (Fig. 3A). After cleavage by *DA1*, the neo-N-terminus of BB exposes a tyrosine residue, which is a potential signal of a type II N-degron (Dissmeyer *et al.*, 2018). Truncated BB proteins (Y61-BB) are rapidly degraded, but stabilized upon the addition of the proteasome inhibitor MG132, in the absence of functional PRT1 or by mutating the exposed tyrosine to a glycine residue (Dong *et al.*, 2017). Upon BL treatment, BB protein levels increase, further illustrating that *BRI1*-mediated phosphorylation of *DA1* reduces its protease activity (Dong *et al.*, 2020).

UBP15 is one of the UBP15 subfamily members of UBPs, characterized by a MYND-type zinc finger domain, which is a known protein–protein interaction domain (Lutterbach *et al.*, 1998). Unlike UBP12 and UBP13, UBP15 acts downstream of *DA1* as its cleavage substrate (Du *et al.*, 2014) (Fig. 3A). Protein levels of UBP15 are higher in plants carrying the *da1-1* allele, which encodes *DA1* proteins with a lower peptidase activity, and UBP15 can be stabilized in the presence of MG132 (Du *et al.*, 2014), suggesting that UBP15 can be cleaved by *DA1* and degraded by the proteasome. Similarly as with BB, levels of UBP15 are also increased after treatment with BL (Dong *et al.*, 2020). UBP15 positively regulates plant organ size, and negatively influences axillary meristem initiation (Liu *et al.*, 2008; Du *et al.*, 2014; Li *et al.*, 2020). In *Arabidopsis*, T-DNA insertion lines of

UBP15 produce smaller plants with narrow and serrated leaves, shorter roots in the seedling stage, smaller flowers, shorter siliques and shorter and slimmer stems (Liu *et al.*, 2008), as is observed in *35S::GFP-DA1* plants (Vanhaeren *et al.*, 2017), but they have slightly more axillary buds (Li *et al.*, 2020). On the contrary, overexpression of *UBP15* results in an increased organ size by stimulating cell proliferation (Liu *et al.*, 2008; Du *et al.*, 2014), a delay in flowering (Liu *et al.*, 2008), similarly as *da1-1* plants (Li *et al.*, 2008; Vanhaeren *et al.*, 2017; Li *et al.*, 2020), and a reduction in axillary buds in both rosette and cauline leaf axils (Li *et al.*, 2020). *OsUBP15*, the rice (*Oryza sativa*) *UBP15* homolog, also plays a positive role in grain growth (Shi *et al.*, 2019). The dominant mutant allele *lg1-D/Osubp15* generates larger grains, and ectopic expression of *OsUBP15* causes a moderate increase in grain width, length and thickness (Shi *et al.*, 2019). In contrast, reducing *OsUBP15* expression levels decreases grain length and thickness (Shi *et al.*, 2019).

The plant-specific TCP transcription factor family shares a 59-amino acid, basic helix-loop-helix (bHLH) motif that allows for DNA binding and protein–protein interactions (Martín-Trillo and Cubas, 2010). Class I TCP proteins, such as TCP14 and TCP15, are proposed to stimulate cell division and growth, whereas class II TCP proteins function as repressors of these processes (Martín-Trillo and Cubas, 2010). TCP14 and TCP15 interact with DA1, DAR1 and DAR2 and higher protein levels of TCP14 and TCP15 are present in *da1-ko1_dar1-1_dar2-1* mutants (Peng *et al.*, 2015). TCP14 and TCP15 act redundantly in regulating organ growth. No obvious phenotype can be observed in *tcp14* mutant plants and only a mild reduction in inflorescence height is visible in *tcp15* mutant lines, but a significant reduction in inflorescence height, pedicel length and internode length is found in *tcp14_tcp15* double mutants (Kieffer *et al.*, 2011). Furthermore, expression of *TCP14* under the control of its native promoter can complement the *tcp14_tcp15* phenotype (Kieffer *et al.*, 2011). In *Arabidopsis*, *TCP14* and *TCP15* are both expressed in all internodes of young inflorescence stems, in young flower pedicels (Kieffer *et al.*, 2011) and in young leaves (Peng *et al.*, 2015), and promote cell proliferation in young stem internodes (Kieffer *et al.*, 2011). *AtTCP15* also represses endoreduplication in *Arabidopsis*. Transgenic *Arabidopsis* plants expressing *TCP15* fused to an EAR repression domain (SRDX) carry trichomes with supernumerary branches and a higher DNA content (Li *et al.*, 2012). On the other hand, inducible expression lines of *TCP15* show a dramatic reduction of trichome branching and lower ploidy levels in leaf cells (Li *et al.*, 2012). TCP15 binds to the promoter regions of *CYCLINA2;3* (*CYCA2;3*) and *RENTINOBLASTOMA-RELATED* (*RBR*), involved in cell cycle progression, and negatively regulates genes encoding positive regulators of endoreduplication, such as *DP-E2F-LIKE 1*, *CDC10 TARGET 1 A*, *PROLIFERATING CELLULAR NUCLEAR ANTIGEN 1*, *WEE1* and *FIZZY-RELATED 2* (Li *et al.*, 2012).

Similar to *UBP15*, TCP14 and TCP15 are cleaved by activated DA1 proteins (Dong *et al.*, 2017) (Fig. 3). Triple *da1-ko1_dar1-1_dar2-1* mutants are dwarfed and their leaves contain small cells with lower endoreduplication levels, phenotypes that are partially restored in the pentuple *tcp14-3_tcp15-3_da1-ko1_dar1-1_dar2-1* mutant (Peng *et al.*, 2015). Likewise, the expression levels of *CYCA2;3* and *RBR* are dramatically elevated in *da1-ko1 dar1-1 dar2-1* mutants but are again lower in *tcp14-3_tcp15-3_da1-ko1_dar1-*

338 *1_dar2-1* plants (Peng *et al.*, 2015). The ability to cleave and potentially destabilize TCP14 and TCP15
339 demonstrates the close link between the DA1 pathway and the core cell cycle. Another class I TCP protein, TCP22,
340 is cleaved by DA1 (Dong *et al.*, 2017). Considering that TCP22 acts redundantly with other class I TCPs in
341 controlling leaf development and endoreduplication, such as TCP7, TCP8, TCP14, TCP15, TCP21, and TCP23
342 (Aguilar-Martínez and Sinha, 2013; Zhang *et al.*, 2019b), and that DA1 also cleaves TCP14 and TCP15, DA1 might
343 potentially target additional class I TCP proteins.

345 **The road ahead: exploring the degradome of proteases**

346 The hunt for protease substrates is a challenging task, mainly because of the momentary nature of the
347 interaction and the, often, destabilizing effect on the substrate. In addition, the catalytic activity of proteases is
348 often strictly controlled by their localization (van Wijk, 2015), inhibitory propeptides (Meyer *et al.*, 2016),
349 multimerization (Dong *et al.*, 2020), proteins that inhibit their active site (Grosse-Holz and van der Hoorn, 2016),
350 environmental triggers (Lam and Zhang, 2012), or post-translational modifications (Gu *et al.*, 2012; Dong *et al.*,
351 2017; Dong *et al.*, 2020), here exemplified by the ubiquitination and phosphorylation of DA1. A successful
352 strategy that can be applied is to compare the N-terminome of protease mutants or mutants in the N-degron
353 pathway compared to a WT situation (Escamez *et al.*, 2016; Zhang *et al.*, 2018a). A potential pitfall of this
354 approach is that such steady-state mutants might display growth differences compared to their controls, as is
355 the case for *da1-1* mutants (Li *et al.*, 2008; Vanhaeren *et al.*, 2017), leading to noise caused by developmental
356 differences, reflected in the proteome. More desirable, an abrupt accumulation of active proteases can generate
357 a much higher and relevant contrast between the proteomes. As such, micro-environmental triggers, such as
358 calcium (Bozhkov *et al.*, 2005; Watanabe and Lam, 2005; Watanabe and Lam, 2011), genetic inducible systems
359 (Corrado and Karali, 2009) or a combination of both can be used to generate a sudden burst of activated
360 proteases, leading to a rapid change and hence contrast in the proteome. This requires however detailed
361 insights into the mechanisms that lead to the activation of the protease. For example DA1, the simultaneous
362 induction a of non-phosphorylatable DA1 (DA1^{S363A,T367SA,T370A}) together with an uncleavable BB (BB^{A60G,Y61G})
363 (Dong *et al.*, 2017) in plants could lead to a strong accumulation of ubiquitinated, active DA1 proteases, leading
364 to the rapid cleavage of its substrates. In addition, to stabilize the cleaved protein fragments, proteasomal
365 inhibitors (e.g. MG-132) can be added to the growth medium prior to the activation of the proteases.

366 Total proteome analyses are however dominated by highly abundant proteins, obscuring the detection
367 of lowly abundant peptides. Enrichment procedures are often required to enhance the detection of lower
368 abundant proteins. Successful *in vivo* purification techniques include tandem affinity purification (TAP) (Van
369 Leene *et al.*, 2015), single step affinity purification (Wendrich *et al.*, 2017) or proximity labeling (Arora *et al.*,
370 2020), coupled to mass spectrometry. Especially the latter, in combination with proteasome inhibitors, has a

huge potential to identify transient interactions. Peptide mixtures can also be specifically enriched for N-terminal peptides by the diagonal chromatography using the COmbined FRActional Diagonal Chromatography (COFRADIC)) sorting procedure (Staes *et al.*, 2017). In this procedure, extracted proteins are chemically modified to reduce and block the cysteine residues, and to acetylate α - and ϵ -amino groups using an N-hydroxysuccinimide-ester of acetate labeled with heavy stable isotopes. The latter allows to mass tag peptides carrying neo-N-termini introduced by the protease. After trypsin digestion, N-terminal peptides can be enriched for through strong cation exchange chromatography, during which most internal peptides are removed (Staes *et al.*, 2017).

Identified interactors and hence candidate substrates can then further be tested for cleavage with in vitro or in vivo methods, reviewed by Fernández-Fernández *et al.* (2019). After proteolytic cleavage, a neo-N-terminus can expose a stabilizing or destabilizing amino acid (Dissmeyer, 2019). Destabilizing residues, such as a basic or aromatic amino acids, can be directly recognized and ubiquitinated by the E3 ligases PRT6 or PRT1, respectively, leading to proteasomal degradation of the substrate (Gibbs *et al.*, 2016). It was shown for BB that after cleavage by DA1, tyrosine was exposed at the neo-N-terminus, leading to its rapid, PRT1-mediated degradation (Dong *et al.*, 2017). If a destabilizing residue is exposed after cleavage, the rapid degradation of the protein can be visualized with a tandem fluorescent timer (Zhang *et al.*, 2019c). The principle of this technique is as follows: the neo-N-terminal protein fragment is expressed behind the coding sequence of ubiquitin and fused to the monomeric red fluorescent mCherry and super folding green fluorescent protein (sfGFP). The sfGFP protein matures at a faster rate (~ 6 min) than the mCherry (a two-step process of ~ 17 min and ~ 30 min) (Khmelniskii *et al.*, 2012). In vivo, endogenous deubiquitination enzymes will cleave off the ubiquitin protein, which leads to the exposure of the destabilizing sequence. As a consequence, more mCherry proteins will be degraded before their maturation compared to the sfGFP (Fig. 4A). If the mCherry/sfGFP ratio is hence lower in WT plants compared to *prt1-1* or *prt6-5* mutants that lack the respective E3 ligases (Garzón *et al.*, 2007; Graciet *et al.*, 2009), this points to the degradation of the protein fragment through the N-degron pathway. Alternatively, cell-free systems in which the target protein is transiently co-expressed with an E3 ligase of interest (García-Cano *et al.*, 2014), or the quantification of the degradation of purified full-length proteins or proteins with a mutated cleavage site in different cell extracts (Cheng *et al.*, 2017; Dong *et al.*, 2017), offer accessible ways to identify the potential degradation of the target proteins.

Given that DA1 cleaves diverse proteins rather than a specific protein family, it would be interesting to investigate if consensus DA1 cleavage sites exist, as has been proposed for METACASPASE 9 (Tsiatsiani *et al.*, 2013). Identifying the cleavage sites of known and novel DA1 substrates could provide novel insights into the DA1 degradome, potentially aided by motif finding (Bailey *et al.*, 2009; Grant *et al.*, 2011).

Back to biology: the effect of endogenous substrate cleavage on cellular processes

Identifying the cleavage substrates of a protease and determining the effect of cleavage on the substrate are the first steps towards the next challenging part: unraveling the biological context of the cleavage event and the subsequent effects on plant development or homeostasis. Once the exact cleavage site of a protease substrate is identified, opportunities arise to characterize the gene of interest in more diverse manners than the classical characterization of mutants or ectopic expression lines. For example, if cleavage of a substrate leads to its rapid degradation, the cleavage sites can be mutated using prime editing (Marzec and Hensel, 2020) to generate proteins with a higher stability in an endogenous context. Alternatively, the mutated transgene can be expressed from its own promoter in the respective mutant background. For substrates that expose a stabilizing residue after cleavage, potentially leading to a change of function, it is possible to generate lines that overexpress these cleaved fragment from their endogenous promoter to study their function and resulting phenotypes.

For DA1, or other proteases that are involved in determining the duration of cell proliferation, it is desired to link their catalytic activity to the core cell cycle. Until now, it remains unclear if DA1 is activated in specific developmental zones of organs to limit the capacity of cells to divide, or if DA1 is activated during certain stages of the cell cycle to limit mitosis. In addition, it remains unknown if the activity of DA1 slows down the potential of cells to divide or whether this happens abruptly and irreversibly. The activity of proteases can be detected with several imaging technologies, such as fluorescence resonance energy transfer sensors, or based on complementation, misfolding or delocalization of fluorescent proteins (Fernández-Fernández *et al.*, 2019). Such advanced confocal imaging techniques, in combination with for example a fluorescent cell cycle phase indicator (Desvoyes *et al.*, 2020), would make it feasible to link the activity of DA1 with the different stages of the cell cycle. Moreover, in the animal field, transcriptional sensors have enabled to track and trace substrate cleavage. For example, a two-component biosensor was developed to study caspase-mediated processing events in *Drosophila* (Tang *et al.*, 2015). Inspired by this, the cleavage sequence of a specific DA1 substrate in *Arabidopsis* can be used to link a GAL4 transcription factor and a strong plasma membrane anchor (Simon *et al.*, 2016), keeping the GAL4 in the cytoplasm. Upon endogenous ubiquitination, DA1 can cleave this linker, which allows GAL4 to migrate to the nucleus, bind an upstream activation system (UAS) and initiate transient expression of genes encoding a nuclear red fluorescent protein (nRFP) and a flippase recombinase (Fig. 4B). The latter can on its turn remove a terminator sequence between a constitutive promoter and a GFP-encoding sequence. This would result in *nRFP* and cytoplasmic *GFP* expression in cells in which DA1 actively cleaves the substrate and constitutive *GFP* expression in all resulting daughter cells, which allows the tracking of the cell lineage after cleavage. In this way, subsequent cell divisions after the cleavage event can be quantified. For nuclear proteins, such as the TCP proteins, a GAL4 protein can be linked with an ethylene-responsive element

binding factor-associated amphiphilic repression (EAR) motif (Kagale and Rozwadowski, 2011), which will inhibit the transcription of nRFP and the flippase (Fig. 4C). When the EAR domain is cleaved off by activated DA1, the inhibition is released and GAL4 can initiate the sensor system as described above (Fig. 4C). In addition, GFP-containing cells or cell lines, in which cleavage by DA1 has occurred, can be isolated through cell sorting of protoplasts. Then, the DNA content can be quantified by flow cytometry to analyze their ploidy levels. In addition, by implementing and adapting the separation of the phases-based activity reporter of kinase (SPARK) (Zhang *et al.*, 2018b), which has been developed in *Drosophila*, the phosphorylation state of DA1 could be visualized during development. With this technology, GFP-based kinase reporters can be phase-separated after kinase activation through multivalent protein–protein interaction, leading to the formation of intense fluorescent droplets (Zhang *et al.*, 2018b).

Conclusions and perspectives for translational research

Besides environmental conditions, intrinsic genetic information greatly determines plant growth and crop yield. Over the last decade, many regulators of organ size have been identified and our knowledge of their molecular mode of action has increased substantially (Gonzalez *et al.*, 2012; Li and Li, 2014; Vanhaeren *et al.*, 2015; Vercruysse *et al.*, 2020). Many of these growth regulators were shown to genetically interact (Vanhaeren *et al.*, 2014) and the molecular links between such seemingly independent pathways are only starting to emerge. For example, the expression of the growth-regulating transcription co-factor AN3/GIF1 was recently found to be controlled by the PPD-KIX complex (Liu *et al.*, 2020b), which in turn regulates organ size (White, 2006; Gonzalez *et al.*, 2015). DA1 also physically interacts with several other proteins, such as BB, UBP15 and BRI1 (Li *et al.*, 2008; Du *et al.*, 2014; Dong *et al.*, 2020), which previously were shown to enhance organ size when mutated or ectopically expressed (Wang *et al.*, 2001; Disch *et al.*, 2006; Liu *et al.*, 2008; Gonzalez *et al.*, 2010). It remains to be explored if additional growth-regulating proteins act up- or downstream of DA1 or regulate its activity. Many members of the DA1 pathway display similar phenotypes in several dicot and monocot species, which demonstrates the value of basic research in *Arabidopsis* and illustrates the unique potential of the DA1 pathway to increase crop yield. It remains however unclear if the molecular mechanisms that fine-tune the activity of DA1 and the cleavage portfolio of DA1 are similarly conserved in such agronomically important species. Regardless, targeting DA1 in crops using genome editing (Ku and Ha, 2020) will be an excellent approach to improve plant yield. Additional phenotypic and biochemical translational research is therefore required to verify the degree of conservation and will eventually reduce the bench-to-field gap.

Acknowledgments

We thank Dr. Annick Bleys for critically reading and improving the manuscript. This work was supported by the

Bijzonder Onderzoeksfonds (BOF08/01M00408). Y.C. was supported by the China Scholarship Council (201604910566), the Bijzonder Onderzoeksfonds (01SC3117) and by Ghent University (BOF-Methusalem projects BOFMET2015000201 & B/09140/02). H.V. is a post-doctoral fellow of Flanders Research Foundation (No. 12V0218N).

Author Contribution

Y.C. and H.V. conceptualized and wrote the manuscript and realized the visualization, D.I. edited the manuscript. H.V. and D.I. supervised the writing.

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753

FIGURES LEGENDS

Fig. 1. DA1-related phenotypes in plants. Arabidopsis *da1-1* mutants produce larger organs, including flowers, siliques, seeds and leaves, and fewer axillary meristems. Constitutive expression of the dominant-negative *DA1* allele (*DA1^{R358K}*) in Arabidopsis mimics the *da1-1* phenotype. In wheat, the *TaDA1* RNAi line produces larger seeds. Likewise, overexpression (OX) of *AtDA1^{R358K}* in *Brassica napus* also results in larger flowers, leaves and seeds. In maize, constitutive expression of the dominant-negative mutation of *ZmDA1* or *ZmDAR1* increases seed size. In contrast, overexpression of *GFP-DA1* in Arabidopsis or of corresponding *DA1* homologs in maize and wheat produce smaller organs, such as flowers and seeds.

Fig. 2. Schematic diagram of the functional domains in DA1, and mutation and post-translational modification sites and their role in regulating the activity of DA1. UIM = ubiquitin-interacting motif, LIM = Lin-11, Isl-1 and Mec-3 domain.

Fig. 3. Model of the DA1 peptidase regulatory network and (de)activation mechanisms of DA1. (A) Schematic overview of the current knowledge of the DA1 pathway in Arabidopsis. The transcription of DA1 is regulated by the transcription factors (TFs) CUC2 and CUC3, which are negatively regulated by BZR1, and through OTU1 by chromatin remodeling. The protease activity of DA1 can be activated by multiple monoubiquitination mediated by the E3 ligases BB and DA2, which is counteracted by UBP12 and UBP13. The protease activity of DA1 is negatively regulated and even overruled by phosphorylation by BAK1/BRI1. Activated DA1 can cleave and destabilize BB and DA2 in a negative feedback loop and cleave downstream regulators of growth, such as UBP15 and the TFs TCP14, TCP22 and TCP15, of which the latter controls the expression of the cell cycle regulators *RBR* and *CYCA2;3*. (B) The effect of ubiquitination, deubiquitination and phosphorylation on the catalytic activity of DA1. Multiple monoubiquitination activates the catalytic activity of DA1, deubiquitination by UBP12 and UBP13 limits this and phosphorylation limits and overrules the protease activity of DA1 by triggering its multimerization. *OTU*: OTUBAIN-LIKE CYSTEINE PROTEASES, *CUC*: CUP-SHAPED COTYLEDON, *BZR*: BRASSINAZOLE-RESISTANT, *BB*: BIG BROTHER, *BAK*: BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE, *BRI*: BRASSINOSTEROID INSENSITIVE, *TCP*: TEOSINTE BRANCHED1, *CYCLOIDEA*, and *PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR*, *UBP*: UBIQUITIN-SPECIFIC PROTEASE, *RBR*: *CYCA2;3*.

Fig. 4. Biochemical characterization of DA1 substrates and in vivo dynamics of DA1 activity and proteolytic cleavage of its substrates. (A) Principles of the tandem fluorescent timer and the stability in Col-0 and *prt1-1* using the BB degron site. Endogenous deubiquitination of the sensor exposes a potential degron signal of the target protein. The differences in protein maturation time of mCherry (slow) and super folding sfGFP (fast) allow

788 the detection of protein degradation by the N-degron pathway. Differences in fluorescent signal between Col-0
789 and mutants of E3 ligases of the N-degron pathway, such as PRT1 (*pri1-1*), indicate changes in degradation rate
790 and can hence pinpoint the causal E3 ligase. Figure adapted from Zhang *et al.* (2019c). (B-C) Cleavage and cell
791 tracker sensor of the DA1 proteolytic activity on (B) cytoplasmic and (C) nuclear substrates. (B) A fusion of a
792 GAL4 transcription factor, a cytoplasmic DA1 substrate (BB) and a strong membrane anchor keeps the GAL4
793 in the cytoplasm. Endogenous activated DA1 can cleave the substrate, allowing GAL4 to migrate to the nucleus
794 and bind upstream activation systems (UAS), which drive the expression of nRFP and a flippase. The first marks
795 cells in which activated DA1 is present, the latter removes a terminator sequence between a constitutive
796 promoter and a GFP-encoding sequence. This results in a constitutive cytoplasmic GFP accumulation in all
797 resulting daughter cells, even when DA1 gets deactivated. (C) Based on the same reporter principle, nuclear
798 proteins, such as transcription factors, can fuse a GAL4 to an EAR domain, hereby inhibiting their activity. When
799 activated, DA1 cleaves off the EAR domain, and the reporter cascade is initiated as described before. Figure
800 adapted from Fernández-Fernández *et al.* (2019).

Arabidopsis thaliana

Zea mays L.

Brassica napus

Triticum aestivum

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A

B

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A

B

C

Fig. 4. Biochemical characterization of DA1 substrates and in vivo dynamics of DA1 activity and proteolytic cleavage of its substrates. (A) Principles of the tandem fluorescent timer and the stability in Col-0 and *prt1-1* using the BB degron site. Endogenous deubiquitination of the sensor exposes a potential degron signal of the target protein. The differences in protein maturation time of mCherry (slow) and super folding sfGFP (fast) allow the detection of protein degradation by the N-degron pathway. Differences in fluorescent signal between Col-0 and mutants of E3 ligases of the N-degron pathway, such as PRT1 (*prt1-1*), indicate changes in degradation rate and can hence pinpoint the causal E3 ligase. Figure adapted from Zhang *et al.* (2019c). (B-C) Cleavage and cell tracker sensor of the DA1 proteolytic activity on (B) cytoplasmic and (C) nuclear substrates. (B) A fusion of a GAL4 transcription factor, a cytoplasmic DA1 substrate (BB) and a strong membrane anchor keeps the GAL4 in the cytoplasm. Endogenous activated DA1 can cleave the substrate, allowing GAL4 to migrate to the nucleus and bind upstream activation systems (UAS), which drive the expression of nRFP and a flippase. The first marks cells in which activated DA1 is present, the latter removes a terminator sequence between a constitutive promoter and a GFP-encoding sequence. This results in a constitutive cytoplasmic GFP accumulation in all resulting daughter cells, even when DA1 gets deactivated. (C) Based on the same reporter principle, nuclear proteins, such as transcription factors, can fuse a GAL4 to an EAR domain, hereby inhibiting their activity. When activated, DA1 cleaves off the EAR domain, and the reporter cascade is initiated as described before. Figure adapted from Fernández-Fernández *et al.* (2019).