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Title Page

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2 Key Message : SUMOylation and anther growth
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11 Title : SUMO proteases OTS1 and 2 control filament elongation through a DELLA-dependent mechanism
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Abstract

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2 During fertilization stamen elongation needs to be synchronised with pistil growth. The phytohormone
3 Gibberellic acid (GAs) promotes stamen growth by stimulating the degradation of growth repressing DELLA
4 proteins. DELLA accumulation is negatively regulated by GAs through the ubiquitin-proteasome system. In
5 *Arabidopsis thaliana* a proportion of DELLAs is also conjugated to the Small Ubiquitin-like Modifier
6 (SUMO) protein, which stabilises DELLAs. Increased DELLA levels occur in the SUMO protease deficient
7 *OVERLY TOLERANT TO SALT 1* and 2 (*ots1 ots2*) double mutants, especially under salt stress conditions.
8 Here we show that *OTS* genes play a redundant role in the control of plant fertility under non-stress
9 conditions. Mutants of *ots1 ots2* display reduced fertility compared with the wild type owing to reduced
10 stamen elongation. Stamen growth, pollination rate and seed production is restored in *ots1 ots2 della*
11 mutants, thus linking OTS1 function to the control of DELLA activity in the context of filament elongation.
12 OTS levels appear to be developmentally regulated as *OTS1/2* transcript upregulation during stamen
13 development overlaps with GAs accumulations. We propose that *OTS* genes enable synchronization of
14 stamen development by facilitating DELLA degradation at a specific developmental stage.
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Keywords

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27 Flower development, SUMOylation, DELLA, Gibberellin, SUMO proteases
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Results and discussion

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32 Gibberellins (GAs) are a class of phytohormones that promote plant growth by stimulating ubiquitination
33 and proteasomal degradation of the growth repressor DELLA (Sun 2010). DELLA degradation initiates
34 when GA binds to the soluble receptor GID1 (Ueguchi-Tanaka et al. 2005; Griffiths et al. 2006; Murase et al.
35 2008). This event stimulates GID1–DELLA binding and recruitment of the E3 ubiquitin-ligase SLEEPY1
36 (Silverstone et al. 2001; Willige et al. 2007; Wang et al. 2009). In contrast to ubiquitination, another covalent
37 modification – SUMOylation – stabilises DELLA. The SUMOylated form of DELLA is proposed to
38 sequester GID1 and consequently facilitate the accumulation of unmodified DELLA in plant cells (Conti et
39 al. 2014; Nelis et al. 2015). The steady state levels of SUMOylated DELLA are dynamically controlled by
40 SUMO proteases *OTS1* and *OTS2* which deSUMOylate DELLA. An increase in the SUMOylated pool of
41 DELLA (as in *ots1-1 ots2-1* double mutant) leads to increased DELLA accumulation and consequent growth
42 restraint under salt stress conditions (when GA accumulation is low). The role of the SUMOylation state of
43 DELLA under non-stress conditions is however poorly understood.
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54 The analysis of the first 10 siliques along the primary stem of *ots1-1 ots2-1* double mutant plants revealed a
55 significantly reduced number of seeds per silique in *ots1-1 ots2-1* double mutant plants compared with wild-
56 type (Figure 1a). This phenotype was completely restored in *ots1-1 ots2-1* plants transformed with the
57 construct *pOTS1::OTS1::GFP* (Online Resource 1) or by external GA applications (Figure 1b). DELLA
58 activity is responsible for the reduced fertility of *ots1-1 ots2-1* mutants as the average number of seeds per
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1 silique was restored to wild-type levels in four *ots1-1 ots2-1 della* combinations (Figure 1a). Unexpectedly,
2 triple mutants of *ots1-1 ots2-1 rgl2-1* produced chlorotic leaves and were not viable, suggesting a specific
3 and redundant role for *OTS1*, *OTS2* and *RGL2* in leaf greening (Online Resource 2).
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5 To gain insights into the role of *DELLA*, *OTS1* and *OTS2* genes during *Arabidopsis* reproduction, we
6 compared the flower morphology of *ots1-1 ots2-1* with *ots1-1 ots2-1 della* mutant plants. In wild-type
7 flowers pollination occurs at stage 13 of flower development (Smyth et al. 1990), when the length of anther
8 filaments exceeds that of the gynoecium and anther dehiscence occurs (Figure 1c). The flowers of *ots1-1*
9 *ots2-1* mutants at stage 13 had anther filaments that did not fully elongate to reach the stigma, and only a
10 partial elongation was observed by stage 14 when some pollen was deposited onto the stigma. Anther
11 elongation appeared to be fully restored in the *ots1-1 ots2-1 rga-100* triple mutants. We confirmed
12 significant differences in filament elongation between wild type and mutants of *ots1-1 ots2-1* at flower stage
13 13 and 14 (Figure 1d). In contrast, stage 12 filament length was similar in all the genotypes analysed,
14 suggesting that OTSs are required during the rapid elongation phase between stage 12 and 13. Also, no
15 similar growth defects were observed in the pistil of *ots1-1 ots2-1* mutants (Figure 1d). Mutations in any of
16 the *DELLA* genes rescued the defects in filaments growth of *ots1-1 ots2-1*, indicating that OTSs promote
17 filament growth via DELLA inactivation (Figure 1d and Online Resource 3).
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28 DELLA are involved in different aspects of plant fertility, including pollen development (Tyler et al. 2004;
29 Plackett et al. 2014). We found no defects in pollen germination in *ots1-1 ots2-1* mutant plants (Online
30 Resource 4), suggesting that pollen viability is not altered in absence of *OTS1* and *OTS2* function. OTS may
31 thus control fertility by synchronizing stamen growth with pistil through DELLA deactivation. GAs control
32 stamen development and act during the early phase of filament elongation (Cheng et al. 2009). Transcript
33 analysis on samples harvested at specific floral stages showed that upregulation of *OTS1* and *OTS2* genes
34 overlapped with *GA3ox1* (encoding a key GA biosynthetic enzyme) between floral stages 12 and 13 (Figure
35 1e). Thus, *OTSs* accumulation occurs immediately before fertilization, mimicking the pattern of GA
36 accumulation (Hu et al. 2008).
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44 SUMOylation of DELLA restrains growth independent of GA accumulation whereas DELLA degradation
45 relies on GA-activated ubiquitination. DELLA deSUMOylation during floral stages 12-13 may promote
46 their degradation. Global accumulation of bioactive GAs is not altered in *ots1-1 ots2-1* mutants as compared
47 with the wild type (Conti et al. 2014). Furthermore, we found no obvious alterations in *GA3ox1* transcript
48 accumulation in *ots1-1 ots2-1* mutants at stages 12 and 13 (Figure 1e). This indicates that endogenous
49 bioactive GA is not sufficient to reset the physiological rate of filament growth of *ots1-1 ots2-1* mutants.
50 Whether SUMOylated DELLA is somewhat resistant to the GA-mediated proteasome destruction is an
51 interesting question for future research. Reduced filament elongation is also associated with defects in the
52 Jasmonate (JA) pathway (Stintzi and Browse 2000). GA and JA signalling pathways exhibit different points
53 of interactions through DELLA (Cheng et al. 2009; Hou et al. 2010; Qi et al. 2014). Exogenous applications
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of JA could rescue the reduced fertility of *ots1-1 ots2-1* (Figure 1b). External JA can thus disengage SUMOylated DELLA action and reactivate growth-promoting processes downstream of DELLA.

Besides regulating different steps of GA signalling (Conti et al. 2014; Kim et al. 2015), SUMOylation controls several responses towards abiotic stress, which usually negatively impact on fertility (Lois et al. 2003; Miura et al. 2007; Su et al. 2013). Thus, a detailed comprehension of the mechanisms underlying reproductive organs differentiation is required to improve crop yield under different environmental conditions.

Author Contribution

LC, RB and CT designed the research. AC, RB, MG and LC conducted experiments. AS contributed new reagents. LC wrote the manuscript with AC and RB. All authors read and approved the manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest.

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40 41 42 43 44 45 46 47 48 49 **Figure Legends**

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52 Figure 1. *OTS1* and *OTS2* redundantly control filament length and fertility of Arabidopsis. **a** Seed yields in
53 different genetic backgrounds. Bars indicate the average number of seeds in each silique at the indicated
54 node positions (1 is the oldest silique). *n* = 10 independent plants for each genotype. Error bars = SE. **b** as in
55 **a**, after treatment of *ots1-1 ots2-1* plants with hormones GA or JA or mock. **c** Stereomicroscopy images of
56 flowers at stage 13 and 14 in wild-type, *ots1-1 ots2-1* and *ots1-1 ots2-1 rga-100* genetic backgrounds. Stage
57 12 is reported in the insets. At stage 13, pollen grains are visible on the top of pistil in wild-type and *ots1-1*
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1 *ots2-1 rga-100* plants; anther filaments do not fully elongate in *ots1-1 ots2-1* mutant flowers. Scale bar = 0,5
2 mm **d** Anther filament and pistil length in wild-type, *rga-100*, *ots1-1 ots2-1* and *ots1-1 ots2-1 rga-100*
3 flowers at stage 12, 13 and 14. n = 14 measurements from at least 5 independent plants / genotype. Error bars
4 = SE. Student's t test P values ≤ 0.001 (*) **e** qRT-PCR analysis performed on flowers at stage 12 and 13. Left
5 panel, accumulation of *OTS1* and *OTS2* transcripts. Right panel, accumulation of *GA3ox1* transcript. At each
6 time point, values represent fold change variations relative to stage 12 in wild type. *ACT2* expression was
7 used for normalization; error bars represent SD of three biological replicates.
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15 **Electronic supplementary material**

16 17 Online resource 1

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20 Seed number in wild-type, *ots1-1 ots2-1* and *ots1-1 ots2-1* mutant plants transformed with a construct
21 carrying the *OTS1* gene under the control of its promoter. These lines were included in the same experiment
22 reported in Fig. 1. The results of wild type and *ots1 ots2* are therefore duplicated here for clarity.
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28 Picture of wild-type and *ots1-1 ots2-1 rgl2-1* plants grown under in vitro conditions on standard Murashige
29 and Skoog medium for 21 days.
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35 Ratio between pistil and filament length in wild-type and mutant plants at stage 13 of flower development.
36 Self-fertilization is impaired when the value is higher than 1 as in *ots1-1 ots2-1* mutant flowers. Shown are
37 seven independent measurements of flowers at stage 13 from at least 3 different plants of the indicated
38 genotypes.
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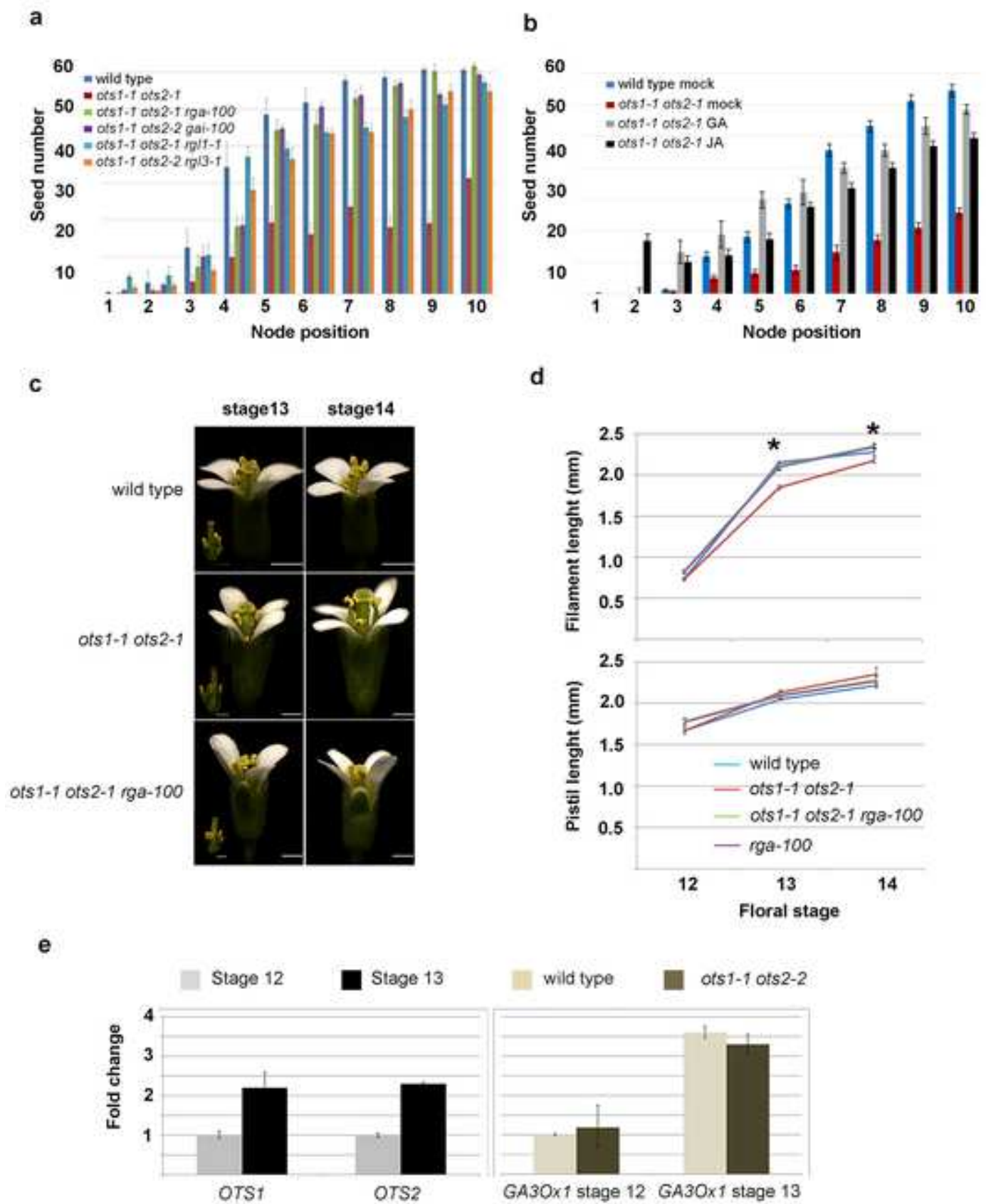
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45 Pollen grains germination in wild-type and *ots1-1 ots2-1* genetic background. Shown is the average
46 percentage of germination in 10 replicated slides. Error bars = SE. N = 300 - 500 pollen grains analysed.
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51 52 Material and Methods

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