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(71) Applicants: **INSTITUTE OF GENETICS AND DEVELOPMENTAL BIOLOGY** [CN/CN]; Chinese Academy of Sciences, Beijing, 100101 (CN). **PLANT BIOSCIENCE LIMITED** [GB/GB]; Norwich Research Park, Colney Lane, Norwich Norfolk NR4 7UH (GB).

(72) Inventors: **LI, Yunhai**; Chinese Academy of Sciences, Beijing, 100101 (CN). **XIA, Tian**; Chinese Academy of Sciences, Beijing, 100101 (CN). **LI, Na**; Chinese Academy of Sciences, Beijing, 100101 (CN). **DUMENIL, Jack**; Department of Cell and Developmental Biology, John Innes Centre, Norwich Norfolk NR4 7UH (GB). **BEVAN, Michael**; Department of Cell and Developmental Biology, John Innes Centre, Norwich Norfolk NR4 7UH (GB).

(74) Agents: **SUTCLIFFE, Nicholas** et al.; Mewburn Ellis LLP, 33 Gutter Lane, London Greater London EC2V 8AS (GB).

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(54) Title: METHODS OF MODULATING SEED AND ORGAN SIZE IN PLANTS

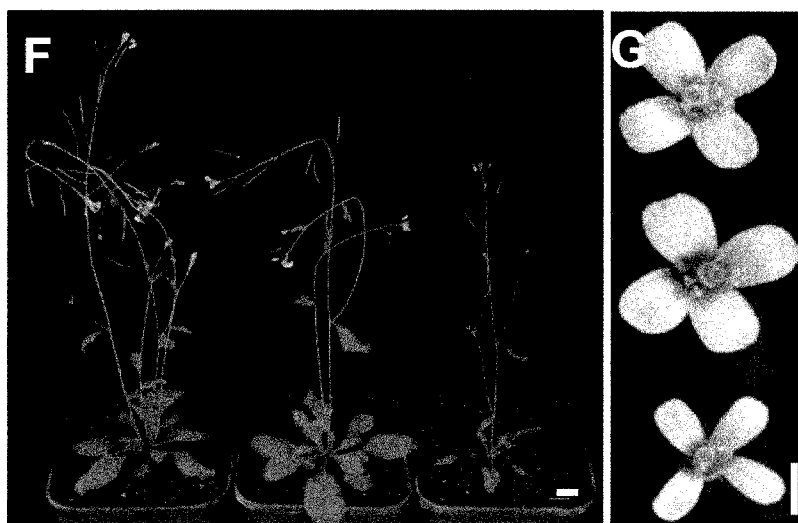


Figure 2

(57) Abstract: This invention relates to a plant E3 ubiquitin ligase (termed DA2) which acts synergistically with DA1 to control seed and organ size. Methods of increasing plant yield are provided that comprise reducing the expression or activity of DA2 in a plant that is deficient in DA1 expression or activity. Plants with increased yield and methods of producing such plants are also provided.



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Methods of Modulating Seed and Organ Size in PlantsField of Invention

This invention relates to methods of altering the size of the seeds and organs of plants, for example to improve plant yield.

Background of Invention

The size of seeds and organs is an agronomically and ecologically important trait that is under genetic control (Alonso-Blanco, C. *PNAS USA* 96, 4710-7 (1999); Song, X.J. *Nat Genet* 39, 623-30 (2007); Weiss, J. *Int J Dev Biol* 49, 513-25 (2005); Dinnyen, J.R. *Development* 131, 1101-10 (2004); Disch, S. *Curr Biol* 16, 272-9 (2006); *Science* 289, 85-8 (2000); Horiguchi, G. *Plant J* 43, 68-78 (2005); Hu, Y. *Plant J* 47, 1-9 (2006); Hu, Y. *Plant Cell* 15, 1951-61 (2003); Krizek, B.A. *Dev Genet* 25, 224-36 (1999); Mizukami, Y. *PNAS USA* 97, 942-7 (2000); Nath, U. *Science* 299, 1404-7 (2003); Ohno, C.K. *Development* 131, 1111-22 (2004); Szecsi, J. *Embo J* 25, 3912-20 (2006); White, D.W. *PNAS USA* 103, 13238-43 (2006); Horvath, B.M. *Embo J* 25, 4909-20 (2006); Garcia, D. *Plant Cell* 17, 52-60 (2005).

The final size of seeds and organs is constant within a given species, whereas interspecies seed and organ size variation is remarkably large, suggesting that plants have regulatory mechanisms that control seed and organ growth in a coordinated and timely manner. Despite the importance of seed and organ size, however, little is known about the molecular and genetic mechanisms that control final organ and seed size in plants.

The genetic regulation of seed size has been investigated in plants, including in tomato, soybean, maize, and rice, using quantitative trait locus (QTL) mapping. To date, in the published literature, two genes (Song, X.J. *Nat Genet* 39, 623-30 (2007); Fan, C. *Theor. Appl. Genet.* 112, 1164-1171 (2006)), underlying two major QTLs for rice grain size, have been identified, although the molecular mechanisms of these genes remain to be elucidated. In *Arabidopsis*, eleven loci affecting seed weight and/or length in crosses between the accessions Ler and Cvi, have been mapped (Alonso-Blanco, 1999 *supra*), but the corresponding genes have not been identified. Recent studies have revealed that AP2 and ARF2 are involved in control of seed size. Unfortunately, however, ap2 and arf2 mutants have lower fertility than wild type (Schruoff, M.C. *Development* 137, 251-261 (2006); Ohto, M.A. *PNAS USA* 102, 3123-3128 (2005); Jofuku, K.D. *PNAS USA* 102, 3117-3122 (2005)). In addition, studies using mutant plants have identified several positive and negative regulators that

influence organ size by acting on cell proliferation or expansion {Krizek, B.A. *Dev Genet* 25, 224-36 (1999); Mizukami, Y. *Proc Natl Acad Sci U S A* 97, 942-7 (2000); Nath, U. *Science* 299, 1404-7 (2003); Ohno, C.K. *Development* 131, 1111-22 (2004); Szecsi, J. *Embo J* 25, 3912-20 (2006); White, D.W. *PNAS USA* 103, 13238-43 (2006); Horvath, B.M. *Embo J* 25, 4909-20 (2006); Garcia, D. *Plant Cell* 17, 52-60 (2005). Horiguchi, G. *Plant J* 43, 68-78 (2005); Hu, Y *Plant J* 47, 1-9 (2006) Dinneny, J.R. *Development* 131, 1101-10 (2004)).

Several factors involved in ubiquitin-related activities have been known to influence seed size. A growth-restricting factor, DA1, is a ubiquitin receptor and contains two ubiquitin interaction motifs (UIMs) that bind ubiquitin *in vitro*, and *dal-1* mutant forms large seeds by influencing the maternal integuments of ovules (Li et al., 2008). Mutations in an enhancer of *dal-1* (*EOD1*), which encodes the E3 ubiquitin ligase BIG BROTHER (BB) (Disch et al., 2006; Li et al., 2008), synergistically enhance the seed size phenotype of *dal-1*, indicating that *DA1* acts synergistically with *EOD1/BB* to control seed size. In rice, a quantitative trait locus (QTL) for *GRAIN WIDTH AND WEIGHT2* (*GW2*), encoding an E3 ubiquitin ligase, controls grain size by restricting cell division (Song et al., 2007). A *GW2* homologue in wheat has been identified (Ta-GW2; Bednarek et al 2012). An unknown protein encoded by rice *qSW5/GW5* is required to limit grain size in rice (Shomura et al., 2008; Weng et al., 2008). *GW5* physically interacts with polyubiquitin in a yeast two-hybrid assay, suggesting that *GW5* may be involved in the ubiquitin-proteasome pathway (Weng et al., 2008). However, it is not clear whether these two factors act in maternal and/or zygotic tissues in rice.

Identification of further factors that control the final size of both seeds and organs will not only advance understanding of the mechanisms of size control in plants, but may also have substantial practical applications for example in improving crop yield and plant biomass for generating biofuel.

Summary of Invention

The present inventors have identified a plant E3 ubiquitin ligase (termed DA2) which regulates the final size of seeds and organs by restricting cell proliferation in the integuments of developing seeds. DA2 was unexpectedly found to act synergistically with DA1 and independently of EOD1 to control seed and organ size. The targeting of DA2 and DA1 and/or EOD1 may therefore be useful in improving plant yield.

An aspect of the invention provides a method of increasing the yield of a plant comprising;

reducing the expression or activity of a DA2 polypeptide
5 within cells of the plant,
wherein the plant is deficient in DA1 expression or activity.

Another aspect of the invention provides a method of increasing the yield of a plant comprising;

10 reducing the expression or activity of a DA2 polypeptide
within cells of the plant,
wherein the plant is deficient in EOD1 expression or activity.

Another aspect of the invention provides a method of increasing the yield of a plant comprising;

15 reducing the expression or activity of a DA2 polypeptide
within cells of said plant,
wherein the plant is deficient in DA1 and EOD1 expression or activity.

20 Another aspect of the invention provides a method of increasing the yield of a plant comprising;

reducing or abolishing the expression or activity of a DA2 polypeptide within cells of said plant, and;

25 i) reducing or abolishing the expression or activity of a DA1 polypeptide within said cells,
ii) reducing or abolishing the expression or activity of EOD1 within said cells, and/or
iii) expressing a dominant-negative DA polypeptide within said
30 cells.

Another aspect of the invention provides a method of producing a plant with an increased yield comprising:

35 providing a plant cell that is deficient in the expression or activity of DA1, EOD1 or both DA1 and EOD1,

incorporating a heterologous nucleic acid which abolishes or suppresses the expression or activity of a DA2 polypeptide into the plant cell by means of transformation, and;

regenerating the plant from one or more transformed cells.

40 Brief Description of Drawings

Figure 1 shows seed and organ size in the *da2-1* mutant. 1A shows the

projective area of Col-0, *da2-1* and *35S::DA2#1* seeds. The seeds were classified into three groups (>0.13 , $0.12-0.13$ and <0.12 mm²).

Values for each group are expressed as a percentage of the total seed number analyzed. 1B shows the seed number per silique for Col-

5 0, *da2-1* and *35S::DA2#1*. Siliques (from the fourth silique to the tenth silique) on main stem were used to measure seed number per silique. 1C shows seed weight per plant for Col-0, *da2-1* and *35S::DA2#1*. 1D shows seed number per plant for Col-0, *da2-1* and *35S::DA2#1*. 1E shows the height of Col-0, *da2-1* and *35S::DA2#1*
 10 plants. Values (B-E) are given as mean $\pm$ SE relative to the wild-type value, set at 100%. **, $P<0.01$ and *, $P<0.05$ compared with the wild type (Student's *t*-test). Bars: F, 1cm; G, 1mm

Figure 2 shows 4-d-old plants (F) of Col-0 (left), *da2-1* (middle)
 15 and *35S::DA2#1* (right) and flowers (G) of Col-0 (top), *da2-1* (middle) and *35S::DA2#1* (bottom).

Figure 3 shows that *DA1* and *DA2* act synergistically to control seed size. 3A shows dry seeds of Col-0, *dal-1*, *da2-1* and *dal-1 da2-1*.

20 3B shows 10-day-old seedlings of Col-0, *da2-1*, *dal-1* and *dal-1 da2-1* (from left to right). 3C shows Seed weight of Col-0, *dal-1*, *da2-1* and *dal-1 da2-1*. 3D shows seed weight of Col-0, *dal-kol*, *da2-1* and *dal-kol da2-1*. Values are given as mean $\pm$ SE relative to the respective wild-type values, set at 100%. **, $P<0.01$ and *, $P<0.05$
 25 compared with the wild type (Student's *t*-test). Bars: A, 0.1mm; B, 1m

Figure 4 shows that *DA1* and *DA2* act synergistically to control seed size. Upper left panel shows cotyledon area of 10-day-old Col-0, *dal-1*, *da2-1* and *dal-1 da2-1* seedlings. Upper right panel shows cotyledon area of 10-day-old Col-0, *dal-kol*, *da2-1* and *dal-kol da2-1* seedlings. Lower left panel shows the average area of palisade cells in cotyledons of Col-0, *dal-1*, *da2-1* and *dal-1 da2-1* embryos. Lower
 30 right panel shows projective area of Col-0, *dal-1*, *dal-1 da2-1*, *dal-kol da2-1*, *dal-kol dal-1* and *dal-kol dal-1 da2-1* seeds. Values are given as mean $\pm$ SE relative to the respective wild-type values, set at 100%. **, $P<0.01$ and *, $P<0.05$ compared with the wild type (Student's *t*-test). Bars: A, 0.1mm; B, 1m

40 Figure 5 shows that *DA1* and *DA2* act synergistically to control cell proliferation in maternal integuments of developing seeds. (5A-5D) show mature Ovules of Col-0, *dal-1*, *da2-1* and *dal-1 da2-1*

respectively. The *da2-1* mutation synergistically enhances the ovule size of *dal-1*.

Figure 6 shows (left panel) the projective area of Col-0 × Col-0 (c/c) F1, *da2-1* × *da2-1* (d2/d2) F1, Col-0 × *da2-1* (c/d2) F1, and *da2-1* × Col-0 (d2/c) F1 seeds and (middle panel) the projective area of Col-0 × Col-0 (c/c) F1, *dal-kol da2-1* × *dal-kol da2-1* (dd/dd) F1, Col-0 × *dal-kol da2-1* (c/dd) F1, *dal-kol da2-1* × Col-0 (dd/c) F1 seeds. Right panel shows projective seed area after pollination of *dal-kol/+ da2-1/+* plants with *dal-kol da2-1* double mutant pollen leading to the development of *dal-kol/+ da2-1/+* (a), *dal-kol/+ da2-1 da2-1* (b), *dal-kol/dal-kol da2-1/+* (c) and *dal-kol da2-1* (d) embryos within *dal-kol/+ da2-1/+* seed coats. Projective area of individual seeds from *dal-kol/+ da2-1/+* plants fertilized with *dal-kol da2-1* double mutant pollen was measured. These seeds were further genotyped for *dal-kol* and *da2-1* mutations. The data shows that *dal-kol* and *da2-1* mutations are not associated with variation in the size of these seeds ($P > 0.05$, Student's *t*-test). Values are given as mean ± SE relative to the respective wild-type values, set at 100%. **, $P < 0.01$ compared with the wild type (Student's *t*-test). Bars: A-D, 0.5 mm.

Figure 7 shows (left panel) the projective area of Col-0, *dal-1*, *da2-1* and *dal-1 da2-1* mature ovules; (middle panel) the number of cells in the outer integuments of Col-0, *dal-1*, *da2-1* and *dal-1 da2-1* seeds at 6 DAP and 8 DAP; and (right panel) the average length of cells in the outer integuments of Col-0, *dal-1*, *da2-1* and *dal-1 da2-1* seeds at 6 DAP and 8 DAP calculated from the outer integument length and cell number for individual seeds.

Figure 8A shows the *DA2* gene structure. The start codon (ATG) and the stop codon (TAA) are indicated. Closed boxes indicate the coding sequence, open boxes indicate the 5' and 3' untranslated regions, and lines between boxes indicate introns. The T-DNA insertion site (*da2-1*) in the *DA2* gene is shown. Figure 8B shows that the *DA2* protein contains a predicted RING domain.

Figure 9 shows E3 ubiquitin ligase activity of *DA2*. MBP-*DA2* and mutated *DA2* (MBP-*DA2*C59S and MBP-*DA2*N91L) fusion proteins were assayed for E3 ubiquitin ligase activity in the presence of E1, E2 and His-ubiquitin (His-Ub). Ubiquitinated proteins were detected by

immunoblotting (IB) with anti-His antibody (Anti-His) and anti-MBP antibody (Anti-MBP), respectively. The lower arrow indicates MBP-DA2 proteins, and the upper arrow shows ubiquitinated MBP-DA2 proteins.

5 Figure 10 shows the projective area of Col-0, *da2-1*, COM#6, COM#8, and COM#10 seeds (upper panel), where COM is *da2-1* transformed with the DA2 coding sequence driven by its own promoter; petal area of Col-0, *da2-1*, COM#6, COM#8, and COM#10 plants (middle panel) and quantitative real-time RT-PCR analysis of the DA2 gene expression in
10 Col-0, *da2-1*, COM#6, COM#8, and COM#10 seedlings (lower panel). Values (D and E) are given as mean $\pm$ SE relative to the *da2-1* values, set at 100%. **, $P < 0.01$ compared with the *da2-1* mutant (Student's *t*-test).

15 Figure 11 shows expression patterns of DA2. 11A shows quantitative real-time RT-PCR analysis of the DA2 gene expression. Total RNA was isolated from roots (R), stems (S), leaves (L), seedlings (Se) and inflorescences (In). 11B-11N show DA2 expression activity monitored by *pDA2:GUS* transgene expression. Four GUS-expressing lines were
20 observed, and all showed a similar pattern, although they differed slightly in the intensity of the staining. Histochemical analysis of GUS activity in a 4-d-old seedling (11B), a 10-d-old seedling (11C), a floral inflorescence (11D), the developing petals (11E-11G), the developing stamens (11H and 11I), the developing carpels (11J-11L),
25 and the developing ovules (11M and 11N) Bars: B-D, 1mm; E-N, 0.1mm.

Figure 12 shows that DA1 directly interacts with DA2 *in vitro*. GST-DA1, GST-DA1R358K, GST-DA1-UIM, GST-DA1-LIM, GST-DA1-LIM+C and GST-DA1-C were pulled down (PD) by MBP-DA2 immobilized on amylose resin
30 and analyzed by immunoblotting (IB) using an anti-GST antibody.

Figure 13 shows a schematic diagram of DA1 and its derivatives containing specific protein domains. The predicted DA1 protein contains two UIM motifs, a single LIM domain and the C-terminal
35 region.

Figure 14 shows that DA1 interacts with DA2 *in vivo*. *Nicotiana benthamiana* leaves were transformed by injection of *Agrobacterium tumefaciens* GV3101 cells harbouring 35S:Myc-DA1 and 35S:GFP-DA2
40 plasmids. Total proteins were immunoprecipitated with GFP-Trap-A, and the immunoblot was probed with anti-GFP and anti-Myc antibodies, respectively. Myc-DA1 was detected in the immunoprecipitated GFP-DA2

complex, indicating that there is a physical association between DA1 and DA2 in planta.

Figure 15 shows that *da2-1* mutants display increased organ size. 15A shows petal length (PL), petal width (PW), petal area (PA), sepal area (SA), carpel length (CL), long stamen length (LSL) and short stamen length (SSL) of Col-0, *da2-1* and 35S:DA2#1 plants. 15B shows fifth leaf area of Col-0, *da2-1* and 35S:DA2#1 plants. 15C shows weight of Col-0, *da2-1* and 35S:DA2#1 flowers. 15D shows the size of adaxial epidermal cells in the maximal width region of Col-0 and *da2-1* petals. 15E shows the size of palisade cells in the fifth leaves of Col-0 and *da2-1*. The opened flowers (stage 14) were used to measure the size of petals (15A), flower weight (C) and the size of epidermal cells (15D). Values (A-E) are given as mean $\pm$ SE relative to the respective wild-type values, set at 100%. **, $P < 0.01$ compared with the wild type (Student's *t*-test).

Figure 16 shows that DA1 and DA2 act synergistically to control seed size. 16D shows the petal area of Col-0, *dal-kol*, *da2-1* and *dal-kolda2-1* flowers. 16E shows the size of adaxial epidermal cells in the maximal width region of Col-0, *dal-kol*, *da2-1* and *dal-kolda2-1* petals. 16F shows seed weight of Col-0, *eod1-2*, *da2-1* and *eod1-2 da2-1*. 16G shows petal area of Col-0, *eod1-2*, *da2-1* and *eod1-2 da2-1*. The opened flowers (stage 14) were used to measure the size of petals (16D and 16G) and the size of epidermal cells (16E). Values (16D-G) are given as mean $\pm$ SE relative to the respective wild-type values, set at 100%. **, $P < 0.01$ and *, $P < 0.05$ compared with the wild type (Student's *t*-test). Bar: 0.1mm.

Figure 17 shows that overexpression of DA2 restricts organ growth. 17A shows that petal area of Col-0, 35S:DA2#2 and 35S:DA2#4. 17B shows expression levels of DA2 in Col-0, 35S:DA2#2 and 35S:DA2#4 seedlings. Values (A and B) are given as mean $\pm$ SE relative to Col-0 values, set at 100%. **, $P < 0.01$ compared with the wild type (Student's *t*-test).

Figure 18 shows that overexpression of DA2L restricts organ growth. 18A shows 20-day-old plants of Col-0, 35S:DA2L#1, 35S:DA2L#3, 35S:DA2L#4, 35S:DA2L#5, and 35S:DA2L#6. 18B shows 30-day-old plants of Col-0, 35S:DA2L#1, 35S:DA2L#3, 35S:DA2L#4, 35S:DA2L#5, and 35S:DA2L#6. 18C shows RT-PCR analysis of DA2L expression in Col-0, 35S:DA2L#1, 35S:DA2L#3, 35S:DA2L#4, 35S:DA2L#5 and 35S:DA2L#6 seedlings. RT-PCR was performed on first-strand cDNA prepared from

2-week-old seedlings. cDNA was standardized by reference to an *ACTIN2* standard. Bars: A, 1cm, B, 1cm

Figure 19 shows that overexpression of *GW2* restricts seed and organ growth. 19A shows 30 -day-old plants of Col-0, 35S:GW2#1, 35S:GW2#2, 35S:GW2#3, 35S:GW2#6 and 35S:GW2L#7. 19B shows projective area of Col-0, 35S:GW2#1, 35S:GW2#2, 35S:GW2#3, 35S:GW2#6 and 35S:GW2L#7 seeds. 19C shows quantitative real-time RT-PCR analysis of the *GW2* gene expression in Col-0, 35S:GW2#1, 35S:GW2#2, 35S:GW2#3, 35S:GW2#6 and 35S:GW2L#7 seedlings. Values (B) are given as mean $\pm$ SE relative to Col-0 values, set at 100%. **, $P < 0.01$ compared with the wild type (Student's *t*-test). Bar: A, 1cm

Detailed Description of Embodiments of the Invention

This invention relates to methods of altering plant traits which affect yield, such as seed and organ size, by altering the expression or activity of the plant E3 ubiquitin ligase DA2 in combination with the alterations in the expression or activity of DA1 and/or EOD1. Preferably, the expression or activity of DA2 and DA1 is altered in the plant.

The expression or activity DA2 expression may be altered before, at the same time, or after alteration of DA1 and/or EOD1 expression or activity. For example, in some embodiments, the expression or activity of a DA2 polypeptide may be altered in one or more plant cells which already have one of; altered DA1 expression or activity, altered EOD1 expression or activity, or altered DA1 and EOD1 expression or activity.

Provided herein are methods of increasing the yield of plant, for example by increasing organ or seed size, that comprise providing a plant that is deficient in DA1 and/or EOD1 expression or activity and reducing the expression of DA2 in one or more cells of the plant. In other embodiments, the expression or activity of DA1 and/or EOD1 may be reduced in one or more plant cells which have reduced expression or activity of a DA2 polypeptide.

Other methods may comprise reducing the expression of DA2 in one or more cells of the plant and reducing the expression or activity of DA1, EOD1 or both DA1 and EOD1 in one or more cells.

Also provided herein are methods of producing a plant with increased yield relative to wild-type plant that comprise;

(a) incorporating into a plant cell by means of transformation
(i) a first heterologous nucleic acid which reduces the
expression of a DA2 polypeptide,

(ii) a second heterologous nucleic acid which reduces
the expression of one of a DA1 polypeptide and a EOD1
polypeptide, and optionally,

(iii) a third heterologous nucleic acid which reduces the
expression of the other of a DA1 polypeptide and a EOD1
polypeptide, and

(b) regenerating the plant from one or more transformed
cells.

Other methods of producing a plant with increased yield may
comprise:

providing a plant cell that is deficient in DA1 and/or EOD1
expression or activity, preferably DA1 activity,

incorporating a heterologous nucleic acid which reduces the
activity or expression of a DA2 polypeptide into the plant cell by
means of transformation, and;

regenerating the plant from the transformed cell.

Following regeneration, a plant with reduced activity or expression
of a DA2 polypeptide and reduced activity or expression of DA1
and/or EOD1 relative to the wild type plant may be selected.

The combination of reduced DA2 expression and reduced DA1 and/or
EOD1 expression synergistically increase the size of the seeds
and/or organs of the plant, thereby increasing the plant yield.

One or more yield-related traits in the plant may be improved by the
combination of reduced DA2 expression or activity and reduced DA1
and/or EOD1 expression or activity. For example, one or more of
life-span, organ size and seed size may be increased in the plant
relative to control or wild-type plants in which expression of the
DA2 polypeptide has not been reduced.

Expression or activity of DA2, DA1 or EOD1 may be reduced in the
methods described herein by at least 50% relative to the wild-type
plant, at least 60%, at least 70%, at least 80%, at least 90%, at
least 95% or at least 98%. In some preferred embodiments, expression
or activity is reduced to zero or substantially zero (i.e.
expression or activity is abolished).

Methods of the invention comprise altering the expression or activity of a DA2 polypeptide in one or more cells of a plant.

DA2 polypeptides are E3 ubiquitin ligases found in plants. DA2 polypeptides whose expression or activity is reduced as described herein may comprise a RING domain (Stone, S.L. et al. (2005)), preferably a C5HC2, C5NC2 or C5TC2 RING domain. A suitable RING domain may consist of the amino acid sequence of SEQ ID NO: 1;

10 C(X)₂C(X)₁₁CC(X)₄CX₂CX₇(H/N/T)X₆CX₂C. (SEQ ID NO: 1)

For example, a suitable RING domain may consist of the amino acid sequence of SEQ ID NO: 2;

15 CPICFL(Y/F)YPSLNRS(K/R)CC(S/M/T/A)K(G/S)ICTECFL(Q/R)MK(P/N/S/V/T/N)(T/P)(H/N/T)(T/S)(A/T/C)(R/Q/K)PTQCP(F/Y)C
(SEQ ID NO:2)

In some embodiments, the H/N/T residue at position 33 in the RING domain of SEQ ID NO: 2 may be T or N.

In some preferred embodiments, a DA2 polypeptide may comprise a RING domain having an amino acid sequence shown in Table 1 (SEQ ID NOS: 3-19), for example Arabidopsis DA2 (SEQ ID NO: 11), Arabidopsis DAL2 (SEQ ID NO: 13) or Rice GW2 (SEQ ID NO: 7) or a variant thereof. For example a RING domain may have the amino acid sequence of residues 59 to 101 of SEQ ID NO: 20 (Pt_GI-224061326.pro), residues 59 to 101 of SEQ ID NO: 21 (Rc_GI-255578534.pro), residues 59 to 101 of SEQ ID NO: 22 (Vv_GI-147817790.pro), residues 59 to 101 of SEQ ID NO: 23 (Gm_GI-356549538.pro), residues 59 to 101 of SEQ ID NO: 24 (At_GI-18411948.pro), residues 61 to 103 of SEQ ID NO: 25 (Ta_GI-408743661.pro), residues 61 to 103 of SEQ ID NO: 26 (Hv_GI-164371454.pro), residues 61 to 103 of SEQ ID NO: 27 (Bd_GI-357140854.pro), residues 62 to 104 of SEQ ID NO: 28 (Os_GI-115445269.pro), residues 63 to 105 of SEQ ID NO: 29 (Sb_GI-242064618.pro), residues 65 to 107 of SEQ ID NO: 30 (Zm_GI-220961719.pro), residues 61 to 103 of SEQ ID NO: 31 (Ta_GI-408743658.pro), residues 43 to 85 of SEQ ID NO: 32 (Bd_GI-357125256.pro), residues 62 to 104 of SEQ ID NO: 33 (Os_GI-218197613.pro), residues 62 to 104 of SEQ ID NO: 34 (Zm_GI-260935347.pro) or residues 62 to 104 of SEQ ID NO: 35 (Sb_GI-242092026.pro).

Further suitable RING domain sequences may be identified using standard sequence analysis techniques as described herein (e.g. Simple Modular Architecture Research Tool (SMART); EMBL Heidelberg, DE).

DA2 polypeptides may further comprise a first consensus domain. The first consensus domain may be located upstream (i.e. on the N terminal side) of the RING domain. A suitable first consensus domain may consist of the amino acid sequence of SEQ ID NO: 36.

Q(Q/Absent)GLY(P/M/N/V/Q/L/V/E) (H/S/N) (P/K/R) D(I/V) D(L/I/H/V/Q) (K/R) KL(R/K) (R/K) LI(V/L) (E/D) (A/S/T) KLAPC
(SEQ ID NO: 36)

In some preferred embodiments, a DA2 polypeptide may comprise a first consensus domain of a DA2 amino acid sequence shown in Table 2, for example residues 20 to 45 of SEQ ID NO: 20, residues 20 to 45 of SEQ ID NO: 21, residues 20 to 45 of SEQ ID NO: 22, residues 20 to 45 of SEQ ID NO: 23, residues 20 to 45 of SEQ ID NO: 24, residues 21 to 46 of SEQ ID NO: 25, residues 21 to 46 of SEQ ID NO: 26, residues 21 to 46 of SEQ ID NO: 27, residues 21 to 46 of SEQ ID NO: 28, residues 21 to 46 of SEQ ID NO: 29, residues 21 to 46 of SEQ ID NO: 30, residues 21 to 46 of SEQ ID NO: 31, residues 4 to 29 of SEQ ID NO: 32, residues 23 to 48 of SEQ ID NO: 33, residues 23 to 48 of SEQ ID NO: 34 or residues 23 to 48 of SEQ ID NO: 35.

A DA2 polypeptide may further comprise a second consensus domain. The second consensus domain may be located downstream (i.e. on the C terminal side) of the RING domain. The second consensus domain may consist of the amino acid sequence of SEQ ID NO: 37.

(N/S) YAVEYRG(V/G) K(T/S) KEE(K/R) (G/S) (V/T/I/F/L/M) EQ(L/I/V/F) EEQ(R/L/K) VIEA(Q/K) (I/M) RMR(H/Q) (K/Q) (E/A)
(SEQ ID NO: 37).

In some preferred embodiments, a DA2 polypeptide may comprise a second consensus domain of an DA2 amino acid sequence shown in Table 2, for example residues 106 to 141 of SEQ ID NO: 20, residues 106 to 141 of SEQ ID NO: 21, residues 106 to 141 of SEQ ID NO: 22, residues 106 to 141 of SEQ ID NO: 23, residues 106 to 141 of SEQ ID NO: 24, residues 107 to 143 of SEQ ID NO: 25, residues 107 to 143 of SEQ ID NO: 26, residues 107 to 143 of SEQ ID NO: 27, residues 108 to 144 of SEQ ID NO: 28, residues 109 to 145 of SEQ ID NO: 29, residues 111 to

147 of SEQ ID NO: 30, residues 107 to 143 of SEQ ID NO: 31, residues 90 to 125 of SEQ ID NO: 32, residues 108 to 143 of SEQ ID NO: 33, residues 108 to 143 of SEQ ID NO: 34 or residues 108 to 143 of SEQ ID NO: 35.

5

Further examples of suitable first and second domain sequences may be identified using standard sequence analysis techniques as described herein (e.g. Simple Modular Architecture Research Tool (SMART); EMBL Heidelberg, DE).

10

In some preferred embodiments, a DA2 polypeptide whose expression or activity is reduced as described herein may comprise a RING Domain of SEQ ID NO: 2, first consensus domain of SEQ ID NO: 36 and a second consensus domain of SEQ ID NO: 37.

15

For example, a DA2 polypeptide may comprise any combination of RING domain sequence, first consensus domain sequence and second consensus domain sequence as set out above.

20

A suitable DA2 polypeptide may comprise an amino acid sequence of any one of SEQ ID NOS 20 to 35 as set out in Table 2 or may be variant of one of these sequences. In some preferred embodiments, a DA2 polypeptide may comprise the amino acid sequence of SEQ ID NO: 28 or 33 (OsgW2), SEQ ID NO: 24 (AtDA2), SEQ ID NO: 25 or SEQ ID NO: 31 (TaGW2) or may be a variant of any one of these sequences which has E3 ubiquitin ligase activity.

25

A DA2 polypeptide which is a variant of any one of SEQ ID NOS: 20 to 35 or other reference DA2 sequence may comprise an amino acid sequence having at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the reference DA2 sequence.

30

35

A DA2 polypeptide which is a variant of any one of SEQ ID NOS: 20 to 35 may further comprise a RING domain having the sequence of SEQ ID NO: 2 a first consensus domain having the sequence of SEQ ID NO: 36 and a second consensus domain having the sequence of SEQ ID NO: 37. Examples of suitable sequences are set out above. In some preferred embodiments, a DA2 polypeptide may comprise the RING domain, first consensus domain and second consensus domain of any one of SEQ ID NOS: 20 to 35.

40

A nucleic acid encoding a DA2 polypeptide may comprise a nucleotide sequence set out in a database entry selected from the group consisting of JN896622.1 GI:408743658 (TaGW2-A); and JN896623.1 GI:408743660 (TaGW2-B) or may be variant of one of these sequences.

In some preferred embodiments, a nucleic acid encoding a DA2 polypeptide may comprise the nucleotide sequence encoding AtDA2, AtDAL2, OsGW2, TaGW2-A or TaGW2-B or may be a variant of any one of these DA2 sequences which encodes a polypeptide which has DA2 activity.

DA2 polypeptides and encoding nucleic acids may be identified in any plant species of interest, in particular a crop plant, such as wheat, barley, maize, rice, soybean, and another agricultural plants, using routine sequence analysis techniques.

Reduction in DA2 expression or activity in a plant is shown herein to synergistically enhance the effect on yield-associated traits in plants of mutations that reduce the activity or expression of DA1. In preferred embodiments, methods described herein may comprise reducing DA2 expression in a plant that is deficient in DA1 expression or activity or reducing both DA1 and DA2 expression in a plant.

DA1 polypeptides are ubiquitin receptors found in plants and are described in detail in Li et al (2008), Wang, et al (2012) and WO2009/047525. DA1 polypeptides whose expression or activity is reduced as described herein may comprise a LIM domain, a conserved C terminal domain and one or more UIM domains.

A LIM domain comprises two Zn finger motifs and may have the amino acid sequence (SEQ ID NO:38);

C(X)₂C(X)₁₆₋₂₃(H/C)(X)_{2/4}(C/H/E)(X)₂C(X)₂C(X)₁₄₋₂₁(C/H)(X)_{2/1/3}(C/H/D/E)X

where X is any amino acid and Zn coordinating residues are underlined.

The Zn coordinating residues in the LIM domain may be C, H, D or E, preferably C.

In some preferred embodiments, a LIM domain may comprise CXXC, HXXCXXCXXC and HxxC motifs, where X is any amino acid. For example, a LIM domain may comprise the amino acid sequence (SEQ ID NO:39);

5 C(X)2C(X)₁₆₋₂₃(H)(X)2(C)(X)2C(X)2C(X)₁₄₋₂₁H(X)2CX

where X is any amino acid and Zn coordinating residues are underlined

10 In some embodiments, a LIM domain may comprise the amino acid sequence of the AtDA1 LIM domain;

CAGCNMEIGHGRFLNCLNSLWHPECFRCYGCSQPISEYEFSTSGNYPFHKACY
(SEQ ID NO: 40; Zn coordinating residues are underlined)

15

Other LIM domains include the LIM domain of an DA1 amino acid sequence shown in Table 3, for example residues 141 to 193 of SEQ ID NO: 41 (Si_GI-514815267.pro), residues 123 to 175 of SEQ ID NO: 42 (Bd_GI-357157184.pro), residues 155 to 207 of SEQ ID NO:

20 43(Br_DA1b.pro), residues 172 to 224 of SEQ ID NO: 44 (Br_DA1a.pro), residues 172 to 224 of SEQ ID NO: 45 (At_GI-15221983.pro), residues 117 to 169 of SEQ ID NO: 46 (Tc_GI-508722773.pro), residues 117 to 169 of SEQ ID NO: 47 (Gm_GI-356564241.pro), residues 121 to 173 of SEQ ID NO: 48 (Gm_GI-356552145.pro), residues 119 to 171 of SEQ ID
25 NO: 49 (Vv_GI-302142429.pro), residues 122 to 174 of SEQ ID NO: 50 (Vv_GI-359492104.pro), residues 125 to 177 of SEQ ID NO: 51 (Sl_GI-460385048.pro), residues 516 to 568 of SEQ ID NO: 52 (Os_GI-218197709.pro), residues 124 to 176 of SEQ ID NO: 53 (Os_GI-115466772.pro), residues 150 to 202 of SEQ ID NO: 54 (Bd_GI-357160893.pro), residues 132 to 184 of SEQ ID NO: 55 (Bd_GI-357164660.pro), residues 124 to 176 of SEQ ID NO: 56 (Sb_GI-242092232.pro), residues 147 to 199 of SEQ ID NO: 57 (Zm_GI-212275448.pro), residues 190 to 242 of SEQ ID NO: 58 (At_GI-240256211.pro), residues 162 to 214 of SEQ ID NO: 59 (At_GI-35
35 145360806.pro), residues 1240 to 1291 of SEQ ID NO: 60 (At_GI-22326876.pro), residues 80 to 122 of SEQ ID NO: 61 (At_GI-30698242.pro), residues 347 to 402 of SEQ ID NO: 62 (At_GI-30698240.pro), residues 286 to 341 of SEQ ID NO: 63 (At_GI-15240018.pro) or residues 202 to 252 of SEQ ID NO: 64 (At_GI-40
40 334188680.pro).

LIM domain sequences may be identified using standard sequence analysis techniques (e.g. Simple Modular Architecture Research Tool (SMART); EMBL Heidelberg, DE).

5 In addition to a LIM domain, a DA1 protein may further comprise a carboxyl terminal region having an amino acid sequence at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% amino acid identity to the sequence of residues 198 to 504 of SEQ ID NO: 41, residues 180 to 487 of SEQ ID NO: 42, residues 212 to 514 of SEQ ID NO: 43, residues 229 to 532 of SEQ ID NO: 44, residues 229 to 532 of SEQ ID NO: 45, residues 174 to 478 of SEQ ID NO: 46, residues 174 to 474 of SEQ ID NO: 47, residues 178 to 478 of SEQ ID NO: 48, residues 176 to 462 of SEQ ID NO: 49, residues 179 to 482 of SEQ ID NO: 50, residues 182 to 486 of SEQ ID NO: 51, residues 573 to 878 of SEQ ID NO: 52, residues 181 to 486 of SEQ ID NO: 53, residues 207 to 512 of SEQ ID NO: 54, residues 189 to 491 of SEQ ID NO: 55, residues 181 to 486 of SEQ ID NO: 56, residues 204 to 508 of SEQ ID NO: 57, residues 247 to 553 of SEQ ID NO: 58, residues 219 to 528 of SEQ ID NO: 59, residues 1296 to 1613 of SEQ ID NO: 60, residues 128 to 450 of SEQ ID NO: 61, residues 404 to 702 of SEQ ID NO: 62, residues 343 to 644 of SEQ ID NO: 63 or residues 256 to 587 of SEQ ID NO: 64.

The carboxyl terminal region of the DA1 protein may comprise the metallopeptidase motif HEMMH (SEQ ID NO: 65).

The carboxyl terminal region may further comprise a EK(X)₃R(X)₄SEEQ (SEQ ID NO: 66) or EK(X)₃R(X)₄SEQ (SEQ ID NO: 67) motif positioned between the LIM domain and HEMMH motif.

30 In addition to a LIM domain and a conserved carboxyl terminal region, a DA1 protein may comprise a UIM1 domain and a UIM2 domain. The UIM1 and UIM2 domains may be located between the N terminal and the LIM domain of the DA1 protein.

35 A UIM1 domain may consist of the sequence of SEQ ID NO: 68 and a UIM2 domain may consist of the sequence of SEQ ID NO: 69.

40 p---pLpbAl pb.Sbp-.pp p (SEQ ID NO: 68)

p---pLpbAl pb.Sbp-spp p (SEQ ID NO: 69)

wherein;

p is a polar amino acid residue, for example, C, D, E, H, K, N, Q, R, S or T;

b is a big amino acid residue, for example, E, F, H, I, K, L, M, Q, R, W or Y;

5 s is a small amino acid residue, for example, A, C, D, G, N, P, S, T or V;

l is an aliphatic amino acid residue, for example, I, L or V;

. is absent or is any amino acid, and

- is any amino acid.

10

Further examples of UIM1 and UIM2 domain sequences may be identified using standard sequence analysis techniques as described herein (e.g. Simple Modular Architecture Research Tool (SMART); EMBL Heidelberg, DE).

15

In some preferred embodiments, a DA1 polypeptide may comprise;

a LIM domain of SEQ ID NO: 39,

a C terminal region having at least 20% sequence identity to residues 229 to 532 of SEQ ID NO: 45 or the equivalent region of
20 any one of SEQ NOS 41 to 44 or 46 to 64, as set out above and comprising a EK(X)₉R(X)₄SEQ or EK(X)₈R(X)₄SEQ motif and a HEMMH motif,

a UIM domain of SEQ ID NO:66, and

a UIM domain of SEQ ID NO:67.

25

A DA1 protein may comprise an amino acid sequence of a plant DA1 protein shown in Table 3 (SEQ ID NOS: 41 to 64) or may be a homologue or variant of one of these sequences which has DA1 activity. For example, a DA1 polypeptide may comprise an amino acid
30 sequence shown in Table 3 (SEQ ID NOS: 41 to 64) or may be variant of one of these sequences which has DA1 activity.

For example, a DA1 polypeptide may comprise an amino acid sequence of AtDA1, AtDAR1, AtDAR2, AtDAR3, AtDAR4, AtDAR5, AtDAR6, AtDAR7, BrDA1a, BrDA1b, BrDAR1, BrDAR2, BrDAR3-7, BrDAL1, BrDAL2, BrDAL3, OsDA1, OsDAR2, OsDAL3, OsDAL5, PpDAL1, PpDAL2, PpDAL3, PpDAL4, PpDAL5, PpDAL6, PpDAL7, PpDAL8, SmDAL1, SmDAL2 or ZmDA1, preferably AtDA1, AtDAR1 BrDA1a, BrDA1b, OsDA1 or ZmDA1 or a homologue or variant of one of these sequences.

40

In some preferred embodiments, a DA1 polypeptide may comprise the amino acid sequence of AtDA1 (AT1G19270; NP_173361.1 GI: 15221983) or may be variant of this sequence which has DA1 activity.

Other DA1 protein sequences which include the characteristic features set out above may be identified using standard sequence analysis tools. A skilled person is readily able to identify nucleic acid sequences encoding DA1 proteins in any plant species of interest.

A DA1 protein in a plant species of interest may have an amino acid sequence which is a variant of a DA1 protein reference amino acid sequence set out herein.

A DA1 polypeptide which is a variant of a reference DA1 sequence, such as any one of SEQ ID NOS 41 to 64, may comprise an amino acid sequence having at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the reference sequence.

Particular amino acid sequence variants that occur in a plant species may differ from a reference sequence set out herein by insertion, addition, substitution or deletion of 1 amino acid, 2, 3, 4, 5-10, 10-20, 20-30, 30-50, or more than 50 amino acids.

In some embodiments, a DA1 polypeptide which is a variant of the AtDA1 sequence of SEQ ID NO: 45 may comprise a UIM1 domain having the sequence QENEDIDRAIALSLLEENQE (SEQ ID NO: 70) and a UIM2 domain having the sequence DEDEQIARALQESMVVGNSP (SEQ ID NO: 71).

A DA1 polypeptide which is a variant of AtDA1 sequence of SEQ ID NO: 45 may comprise a LIM domain having the sequence:

ICAGCNMEIGHGRFLNCLNSLWHPECFRCYGCSPISEYEFSTSGNYPFHKAC
(SEQ ID NO: 72)

A nucleic acid encoding a DA1 polypeptide may comprise a nucleotide sequence set out in a database entry selected from the group consisting of NM_101785.3 GI:42562170 (AtDA1); NM_001057237.1 GI:115454202 (OsDA1); BT085014.1 GI: 238008663 (ZmDA1) or may be variant of one of these sequences which encodes an active DA1 polypeptide.

In some preferred embodiments, a nucleic acid encoding a DA1 polypeptide may comprise the nucleotide sequence of AtDA1

(NM_101785.3 GI: 42562170), ZmDA1 (BT085014.1 GI: 238008663), OsDA1 (NM_001057237.1 GI:115454202) or may be a variant of any one of these sequences which encodes a polypeptide which retains DA1 activity.

DA1 polypeptides and encoding nucleic acids may be identified in plant species, in particular crop plants, such as wheat, barley, maize, rice, and another agricultural plants, using routine sequence analysis techniques.

In some preferred embodiments, DA1 activity in one or more cells of a plant may be reduced by expression of a dominant-negative DA1 polypeptide in the one or more cells (see for example Li et al (2008); WO2009/047525; Wang et al 2012). A plant expressing a dominant-negative DA1 polypeptide may have a *dal-1* phenotype.

A dominant negative allele of a DA1 polypeptide may comprise a DA1 polypeptide having a mutation, e.g. a substitution or deletion, at a conserved R residue that is located at position 358 of the *A. thaliana* DA1 amino acid sequence, position 333 of the *Z. mays* DA1 amino acid sequence or the equivalent position in another DA1 amino acid sequence. For example, a dominant negative allele of a DA1 polypeptide may comprise a mutation of the conserved R residue at a position equivalent to position 358 of the *A. thaliana* DA1 amino acid sequence or position 333 of the *Z. mays* DA1 amino acid sequence. In preferred embodiments, the conserved R residue may be substituted for K.

The conserved R residue that is located at a position in a DA1 amino acid sequence which is equivalent to position 358 of SEQ ID NO: 45 of *A. thaliana* DA1 or position 333 of the *Z. mays* DA1 of SEQ ID NO: 57 is located at the position within the DA1 amino acid sequence which corresponds to R333 of SEQ ID NO:57 and R358 of SEQ ID NO:45 i.e. it is in the same position relative to the other motifs and domains of the DA1 protein. The conserved R residue is located between the LIM domain and the HEMMH peptidase motif of the C terminal region and is completely conserved in the same sequence context in DA1 proteins. The conserved R residue may be contained in a EK(X)₈R(X)₄SEQ (SEQ ID NO: 66) or EK(X)₈R(X)₄SEQ (SEQ ID NO: 67) motif within the C terminal region.

The conserved R residue may be identified by aligning these conserved C terminal regions using standard sequence analysis and

alignment tools and is identified with an arrow in the sequences of Table 3.

Nucleic acid which encodes a dominant negative allele of a DA protein may be produced by any convenient technique. For example, site directed mutagenesis may be employed on a nucleic acid encoding a DA1 polypeptide to alter the conserved R residue at the equivalent position to R358 of *A. thaliana* DA1 or R333 of the *Zea mays* DA1, for example to K. Reagents and kits for *in vitro* mutagenesis are commercially available.

In some embodiments, a nucleic acid encoding a dominant-negative DA1 polypeptide as described herein may be operably linked to a heterologous regulatory sequence, such as a promoter, for example a constitutive, inducible, tissue-specific or developmental specific promoter. The nucleic acid encoding the dominant-negative DA1 polypeptide may be comprised in one or more vectors. For example, the mutated nucleic acid encoding the dominant-negative allele of a DA1 protein may be further cloned into an expression vector and expressed in plant cells as described below to alter the plant phenotype.

In other embodiments, a mutation may be introduced into an endogenous DA1 nucleic acid in a plant, such that the DA1 polypeptide encoded by the mutant DA1 nucleic acid has dominant-negative activity.

Nucleic acid encoding a dominant-negative DA1 polypeptide may be expressed in the same plant species or variety from which it was originally isolated or in a different plant species or variety (i.e. a heterologous plant).

Reduction or abolition of DA2 expression in a plant is also shown herein to enhance the effect of mutations that reduce the expression or activity of EOD1 on yield-associated traits in plants.

Methods described herein may comprise reducing DA2 expression or activity in a plant that is deficient in EOD1 expression or activity or reducing both DA2 and EOD1 expression or activity in a plant. In preferred embodiments, the plant may also be deficient in DA1 activity or the method may additionally comprise reducing or abolishing DA1 expression in the plant

EOD1 polypeptides are E3 ubiquitin ligases found in plants and are described in detail in Disch et al. (2006), Li et al (2008) and WO2009/047525.

- 5 An EOD1 polypeptide whose expression or activity is reduced as described herein may comprise an EOD domain. A suitable EOD domain may consist of the amino acid sequence of SEQ ID NO: 73;

10 (E/K)RCVICQ(L/M) (K/R/G/T/E)Y(K/R) (R/I) (G/K) (D/N/E) (R/Q/K/L)Q(I
/M/V) (K/N/T/A)L(L/P)C(K/S)H(V/A)YH(S/T/G/A) (E/Q/D/S/G)C(I/G/T/
V) (S/T) (K/R)WL(G/T/S)INK(V/I/A/K)CP(V/I)C (SEQ ID NO: 73)

In some preferred embodiments, an EOD1 polypeptide may comprise a EOD domain having an amino acid sequence of residues 195 to 237 of
15 SEQ ID NO: 74 (Zm_GI-223973923.pro), residues 195 to 237 of SEQ ID NO: 75 (Sb_GI-242042045.pro), residues 195 to 237 of SEQ ID NO: 76 (Zm_GI-226496789.pro), residues 218 to 260 of SEQ ID NO: 77 (Os_GI-222624282.pro), residues 196 to 238 of SEQ ID NO: 78 (Os_GI-115451045.pro), residues 197 to 239 of SEQ ID NO: 79 (Bd_GI-357113826.pro), residues 193 to 235 of SEQ ID NO: 80 (Sl_GI-460410949.pro), residues 187 to 229 of SEQ ID NO: 81 (Rc_GI-255582236.pro), residues 150 to 192 of SEQ ID NO: 82 (Pt_GI-224059640.pro), residues 194 to 236 of SEQ ID NO: 83 (Gm_GI-356548935.pro), residues 194 to 236 of SEQ ID NO: 84 (Gm_GI-356544176.pro), residues 194 to 236 of SEQ ID NO: 85 (Vv_GI-359487286.pro), residues 189 to 231 of SEQ ID NO: 86 (Tc_GI-508704801.pro), residues 192 to 234 of SEQ ID NO: 87 (Pp_GI-462414664.pro), residues 190 to 232 of SEQ ID NO: 88 (Cr_GI-482561003.pro), residues 195 to 237 of SEQ ID NO: 89 (At_GI-22331928.pro) or residues 195 to 237 (Sl_GI-460370551.pro) of SEQ
20 25 30 ID NO: 90, as shown in Table 4.

Further suitable EOD domain sequences may be identified using standard sequence analysis techniques as described herein (e.g.
35 Simple Modular Architecture Research Tool (SMART); EMBL Heidelberg, DE).

A EOD1 polypeptide whose expression or activity is reduced as described herein may comprise an amino acid sequence of any one of
40 SEQ ID NOS 74 to 90 as set out in Table 4. In some preferred embodiments, a EOD1 polypeptide may comprise the amino acid sequence of SEQ ID NO: 89 (AtEOD1) or SEQ ID NOS: 77 or 78 (OsEOD1) or may be

a variant of this sequence which retains E3 ubiquitin ligase activity.

A EOD1 polypeptide which is a variant of any one of SEQ ID NOS: 74 to 90 or other reference EOD1 sequence may comprise an amino acid sequence having at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the reference EOD1 sequence.

A EOD polypeptide which is a variant of any one of SEQ ID NOS: 74 to 90 may further comprise a EOD domain having the sequence of SEQ ID NO: 73. Examples of suitable sequences are set out above.

A nucleic acid encoding a EOD1 polypeptide may comprise a nucleotide sequence set out in a database entry selected from the group consisting of XM_002299911.1 GI:224059639 (PtEOD1); XM_002531864.1 GI:255582235 (RcEOD1); XM_002279758.2 GI:359487285 (VvEOD1); XM_003542806.1 GI:356548934 (GmEOD1a); XM_003540482.1 GI:356544175 (GmEOD1b); XM_002468372.1 GI:242042044 (SbEOD1); NM_001147247.1 GI:226496788 (ZmEOD1); or NP_001030922.1 GI: 79316205 (AtEOD1; At3g63530) or may be variant of one of these sequences.

In some preferred embodiments, a nucleic acid encoding a EOD1 polypeptide may comprise the nucleotide sequence encoding AtEOD1 or OsEOD1 or may be a variant of any one of these sequences which encodes a polypeptide which has EOD1 activity.

EOD1 polypeptides and encoding nucleic acids whose expression or activity is reduced as described herein may be readily identified in any plant species of interest, in particular a crop plant, such as wheat, barley, maize, rice, and another agricultural plants, using routine sequence analysis techniques.

DA2 mutation in plants is also shown herein to synergistically enhance the effect of combinations of DA1 and EOD1 mutations on yield-associated traits in plants.

The methods described herein are not limited to particular plant species and expression or activity of DA2, DA1 and/or EOD1 may be reduced in any plant species of interest, as described herein.

An DA1, DA2 or EOD1 polypeptide in a plant species of interest may have an amino acid sequence which is a variant of a respective DA1, DA2 or EOD1 reference amino acid sequence set out herein. A DA1, DA2 or EOD1 polypeptide which is a variant of a reference sequence set out herein, may comprise an amino acid sequence having at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the reference sequence.

Particular amino acid sequence variants that occur in a plant species may differ from a reference sequence set out herein by insertion, addition, substitution or deletion of 1 amino acid, 2, 3, 4, 5-10, 10-20 20-30, 30-50, or more than 50 amino acids.

A DA1, DA2 or EOD1 nucleic acid in a plant species of interest may have a nucleotide sequence which is a variant of a respective DA1, DA2 or EOD1 reference nucleotide sequence set out herein. For example, variant nucleotide sequence may be a homologue, or allele of a reference DA1, DA2 or EOD1 sequence set out herein, and may differ from the reference DA1, DA2 or EOD1 nucleotide sequence by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, for example 2, 3, 4, 5-10, 10-20 20-30, 30-50, or more than 50, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide. Of course, changes to the nucleic acid that make no difference to the encoded amino acid sequence are included. A DA1, DA2 or EOD1 encoding nucleic acid may comprise a sequence having at least 20% or at least 30% sequence identity with the reference nucleic acid sequence, preferably at least 40%, at least 50%, at least 60%, at least 65%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98%. Sequence identity is described above.

Sequence similarity and identity are commonly defined with reference to the algorithm GAP (Wisconsin Package, Accelrys, San Diego USA). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of GAP may be preferred but other algorithms may be used, e.g. BLAST (which uses the method of Altschul et al. (1990) *J. Mol. Biol.* 215: 405-410), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* 85: 2444-2448), or the Smith-Waterman algorithm (Smith and

Waterman (1981) *J. Mol Biol.* 147: 195-197), or the TBLASTN program, of Altschul et al. (1990) supra, generally employing default parameters. In particular, the psi-Blast algorithm (Nucl. Acids Res. (1997) 25 3389-3402) may be used.

5

Sequence comparison may be made over the full-length of the relevant sequence described herein.

Suitable variant amino acid and nucleotide sequences can be identified in any plant species of interest using standard sequence analysis techniques.

A DA1, DA2 or EOD1 nucleotide sequence which is a variant of a reference DA1, DA2 or EOD1 nucleic acid sequence set out herein, may selectively hybridise under stringent conditions with this nucleic acid sequence or the complement thereof.

Stringent conditions include, e.g. for hybridization of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na₂HPO₄, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na₂HPO₄, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

25

An alternative, which may be particularly appropriate with plant nucleic acid preparations, is a solution of 5x SSPE (final 0.9 M NaCl, 0.05M sodium phosphate, 0.005M EDTA pH 7.7), 5X Denhardt's solution, 0.5% SDS, at 50°C or 65°C overnight. Washes may be performed in 0.2x SSC/0.1% SDS at 65°C or at 50-60°C in 1x SSC/0.1% SDS, as required.

Nucleic acids as described herein may be wholly or partially synthetic. In particular, they may be recombinant in that nucleic acid sequences which are not found together in nature (do not run contiguously) have been ligated or otherwise combined artificially. Alternatively, they may have been synthesised directly e.g. using an automated synthesiser.

The expression of a DA2 nucleic acid and a DA1 and/or EOD1 nucleic acid may be reduced or abolished in one or more cells of a plant by any convenient technique.

40

Methods for reducing the expression or activity of a DA2 polypeptide and a DA1 and/or EOD1 polypeptide in a plant are well-known in the art and are described in more detail below. In some embodiments, the expression of active DA2, DA1 and/or EOD1 polypeptide may be

5 reduced, preferably abolished, by introducing a mutation into the nucleic acid sequence in a plant cell which encodes the polypeptide or which regulate the expression of such a nucleic acid sequence. The mutation may disrupt the expression or function of the DA2, DA1 and/or EOD1 polypeptide. Suitable mutations include knock-out and
10 knock-down mutations. In some embodiments, a mutation may produce a dominant-negative allele of DA1. A plant may then be regenerated from the mutated cell. The nucleic acids may be mutated by insertion or deletion of one or more nucleotides. Techniques for the mutagenesis, inactivation or knockout of target genes are well-known
15 in the art (see for example *In Vitro Mutagenesis Protocols*; *Methods in Molecular Biology* (2nd edition) Ed Jeff Braman; Sambrook J et al. 2012. *Molecular Cloning: A Laboratory Manual* (4th Edition) CSH Press; *Current Protocols in Molecular Biology*; Ed Ausubel et al (2013) Wiley). In some embodiments, mutations may be introduced into
20 a target EOD1, DA2 or DA1 gene by genome editing techniques, for example RNA guided nuclease techniques such as CRISPR, Zinc-finger nucleases (ZFNs) and transactivator-like effector nucleases (TALENs) (Urnov, F.D. et al *Nature reviews. Genetics* 11, 636-646 (2010); Joung, J.K. et al. *Nature reviews. Molecular cell biology* 14, 49-55
25 (2013); Gasiunas, G. et al *PNAS USA* 109, E2579-2586 (2012); Cong, L. et al. *Science* 339, 819-823 (2013)).

Sequence mutations which reduce the expression or activity may include a deletion, insertion or substitution of one or more
30 nucleotides, relative to the wild-type nucleotide sequence, a gene amplification or an increase or decrease in methylation, for example hypermethylation. The one or more mutations may be in a coding or non-coding region of the nucleic acid sequence. Mutations in the coding region of the gene encoding the component may prevent the
35 translation of full-length active protein i.e. truncating mutations, or allow the translation of full-length but inactive or impaired function protein i.e. mis-sense mutations. Mutations or epigenetic changes, such as methylation, in non-coding regions of the gene encoding the component, for example, in a regulatory element, may
40 prevent transcription of the gene. A nucleic acid comprising one or more sequence mutations may encode a variant polypeptide which has reduced or abolished activity or may encode a wild-type polypeptide which has little or no expression within the cell, for example

through the altered activity of a regulatory element. A nucleic acid comprising one or more sequence mutations may have one, two, three, four or more mutations relative to the unmutated sequence.

5 For example, the activity of EOD1 may be reduced, preferably abolished, by introducing a mutation, such as a deletion, insertion or substitution, at a position corresponding to position 44 of SEQ ID NO: 89, for example, an A to T substitution. A position in a EOD1 polypeptide sequence which is equivalent to position 44 of SEQ ID
10 NO: 89 may be identified using standard sequence analysis and alignment tools, as shown in Table 4.

DA2, DA1 and EOD1 coding sequences may be identified in any plant species of interest using standard sequence analysis techniques, for
15 example by comparison with the reference sequences set out herein.

Mutations suitable for abolishing expression of an active DA2, DA1 and/or EOD1 polypeptide will be readily apparent to the skilled person.

20 In some preferred embodiments, a mutation that reduces or abolishes DA2 expression or activity may be introduced into a plant cell that expresses a dominant negative DA1 polypeptide and optionally comprises either i) a heterologous nucleic acid that encodes an EOD1
25 suppressor nucleic acid or ii) a mutation that reduces EOD1 expression or activity.

In some embodiments, the expression of a DA1, DA2 and/or EOD1 polypeptide may be reduced in a plant cell by expressing a
30 heterologous nucleic acid which encodes or transcribes a suppressor nucleic acid, for example a suppressor RNA or RNAi molecule, within cells of said plant. The suppressor RNA suppresses the expression of its target polypeptide (i.e. DA1, DA2 or EOD1) in the plant cells.

Nucleic acids as described herein may be wholly or partially
35 synthetic. In particular, they may be recombinant in that nucleic acid sequences which are not found together in nature (do not run contiguously) have been ligated or otherwise combined artificially. Alternatively, they may have been synthesised directly e.g. using an automated synthesiser.

40 The nucleic acid may of course be double- or single-stranded, cDNA or genomic DNA, or RNA. The nucleic acid may be wholly or partially

synthetic, depending on design. Naturally, the skilled person will understand that where the nucleic acid includes RNA, reference to the sequence shown should be construed as reference to the RNA equivalent, with U substituted for T.

5

"Heterologous" indicates that the gene/sequence of nucleotides in question or a sequence regulating the gene/sequence in question, has been introduced into said cells of the plant or an ancestor thereof, using genetic engineering or recombinant means, i.e. by human
10 intervention. Nucleotide sequences which are heterologous to a plant cell may be non-naturally occurring in cells of that type, variety or species (i.e. exogenous or foreign) or may be sequences which are non-naturally occurring in that sub-cellular or genomic environment of the cells or may be sequences which are non-naturally regulated
15 in the cells i.e. operably linked to a non-natural regulatory element.

The suppression of the expression of a target polypeptide in plant cells is well-known in the art. A suitable suppressor nucleic acid
20 may be a copy of all or part of the target DA1, DA2 and/or EOD1 gene inserted in antisense or sense orientation or both relative to the DA1, DA2 and/or EOD1 gene, to achieve reduction in expression of the target gene. See, for example, van der Krol et al., (1990) *The Plant Cell* 2, 291-299; Napoli et al., (1990) *The Plant Cell* 2, 279-
25 289; Zhang et al., (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020. Further refinements of this approach may be found in WO95/34668 (Biosource); Angell & Baulcombe (1997) *The EMBO Journal* 16, 12:3675-3684; and Voinnet & Baulcombe (1997) *Nature* 389: pg 553.

In some embodiments, the suppressor nucleic acid may be a sense
30 suppressor of expression of the DA1, DA2 and/or EOD1 polypeptide.

A suitable sense suppressor nucleic acid may be a double stranded RNA (Fire A. et al *Nature*, Vol 391, (1998)). dsRNA mediated silencing is gene specific and is often termed RNA interference (RNAi). RNAi is a two-step process. First, dsRNA is cleaved within
35 the cell to yield short interfering RNAs (siRNAs) of about 21-23nt length with 5' terminal phosphate and 3' short overhangs (~2nt). The siRNAs target the corresponding mRNA sequence specifically for destruction (Zamore P.D. *Nature Structural Biology*, 8, 9, 746-750, (2001))

40 siRNAs (sometimes called microRNAs) down-regulate gene expression by binding to complementary RNAs and either triggering mRNA elimination

(RNAi) or arresting mRNA translation into protein. siRNA may be derived by processing of long double stranded RNAs and when found in nature are typically of exogenous origin. Micro-interfering RNAs (miRNA) are endogenously encoded small non-coding RNAs, derived by processing of short hairpins. Both siRNA and miRNA can inhibit the translation of mRNAs bearing partially complementary target sequences without RNA cleavage and degrade mRNAs bearing fully complementary sequences.

Accordingly, the present invention provides the use of RNAi sequences based on the DA1, DA2 and/or EOD1 nucleic acid sequence for suppression of the expression of the DA1, DA2 and/or EOD1 polypeptide. For example, an RNAi sequence may correspond to a fragment of a reference DA2, DA1 or EOD1 nucleotide sequence set out herein or may be a variant thereof.

siRNA molecules are typically double stranded and, in order to optimise the effectiveness of RNA mediated down-regulation of the function of a target gene, it is preferred that the length and sequence of the siRNA molecule is chosen to ensure correct recognition of the siRNA by the RISC complex that mediates the recognition by the siRNA of the mRNA target and so that the siRNA is short enough to reduce a host response.

miRNA ligands are typically single stranded and have regions that are partially complementary enabling the ligands to form a hairpin. miRNAs are RNA sequences which are transcribed from DNA, but are not translated into protein. A DNA sequence that codes for a miRNA is longer than the miRNA. This DNA sequence includes the miRNA sequence and an approximate reverse complement. When this DNA sequence is transcribed into a single-stranded RNA molecule, the miRNA sequence and its reverse-complement base pair to form a partially double stranded RNA segment. The design of microRNA sequences is discussed on John et al, PLoS Biology, 11(2), 1862-1879, 2004.

Typically, the RNA molecules intended to mimic the effects of siRNA or miRNA have between 10 and 40 ribonucleotides (or synthetic analogues thereof), more preferably between 17 and 30 ribonucleotides, more preferably between 19 and 25 ribonucleotides and most preferably between 21 and 23 ribonucleotides. In some embodiments of the invention employing double-stranded siRNA, the molecule may have symmetric 3' overhangs, e.g. of one or two

(ribo)nucleotides, typically a UU or dTdT 3' overhang. Based on the disclosure provided herein, the skilled person can readily design suitable siRNA and miRNA sequences, for example using resources such as siRNA finder (Ambion). siRNA and miRNA sequences can be synthetically produced and added exogenously to cause gene downregulation or produced using expression systems (e.g. vectors). In a preferred embodiment, the siRNA is synthesized synthetically.

Longer double stranded RNAs may be processed in the cell to produce siRNAs (see for example Myers (2003) *Nature Biotechnology* 21:324-328). The longer dsRNA molecule may have symmetric 3' or 5' overhangs, e.g. of one or two (ribo) nucleotides, or may have blunt ends. The longer dsRNA molecules may be 25 nucleotides or longer. Preferably, the longer dsRNA molecules are between 25 and 30 nucleotides long. More preferably, the longer dsRNA molecules are between 25 and 27 nucleotides long. Most preferably, the longer dsRNA molecules are 27 nucleotides in length. dsRNAs 30 nucleotides or more in length may be expressed using the vector pDECAP (Shinagawa et al., *Genes and Dev.*, 17, 1340-5, 2003).

Another alternative is the expression of a short hairpin RNA molecule (shRNA) in the cell. shRNAs are more stable than synthetic siRNAs. A shRNA consists of short inverted repeats separated by a small loop sequence. One inverted repeat is complementary to the gene target. In the cell the shRNA is processed by DICER into a siRNA which degrades the target gene mRNA and suppresses expression. In a preferred embodiment the shRNA is produced endogenously (within a cell) by transcription from a vector. shRNAs may be produced within a cell by transfecting the cell with a vector encoding the shRNA sequence under control of a RNA polymerase III promoter such as the human H1 or 7SK promoter or a RNA polymerase II promoter. Alternatively, the shRNA may be synthesised exogenously (in vitro) by transcription from a vector. The shRNA may then be introduced directly into the cell. Preferably, the shRNA molecule comprises a partial sequence of DA1, DA2 and/or EOD1. For example, the shRNA sequence is between 40 and 100 bases in length, more preferably between 40 and 70 bases in length. The stem of the hairpin is preferably between 19 and 30 base pairs in length. The stem may contain G-U pairings to stabilise the hairpin structure.

siRNA molecules, longer dsRNA molecules or miRNA molecules may be made recombinantly by transcription of a nucleic acid sequence, preferably contained within a vector. Preferably, the siRNA

molecule, longer dsRNA molecule or miRNA molecule comprises a partial sequence of a reference DA2, DA1 or EOD1 nucleotide sequence set out herein or a variant thereof.

5 In other embodiments, the suppressor nucleic acid may be an anti-sense suppressor of expression of the DA1, DA2 and/or EOD1 polypeptide. In using anti-sense sequences to down-regulate gene expression, a nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields
10 RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. See, for example, Rothstein et al, 1987; Smith et al, (1988) *Nature* 334, 724-726; Zhang et al, (1992) *The Plant Cell* 4, 1575-1588, English et al., (1996) *The Plant Cell* 8, 179-188. Antisense technology is also reviewed in Bourque,
15 (1995), *Plant Science* 105, 125-149, and Flavell (1994) *PNAS USA* 91, 3490-3496.

An anti-sense suppressor nucleic acid may comprise an anti-sense sequence of at least 10 nucleotides from a nucleotide sequence is a
20 fragment of a reference DA2, DA1 or EOD1 nucleotide sequence set out herein or a variant thereof.

It may be preferable that there is complete sequence identity in the sequence used for down-regulation of expression of a target
25 sequence, and the target sequence, although total complementarity or similarity of sequence is not essential. One or more nucleotides may differ in the sequence used from the target gene. Thus, a sequence employed in a down-regulation of gene expression in accordance with the present invention may be a wild-type sequence
30 (e.g. gene) selected from those available, or a variant of such a sequence.

The sequence need not include an open reading frame or specify an RNA that would be translatable. It may be preferred for there to be
35 sufficient homology for the respective anti-sense and sense RNA molecules to hybridise. There may be down regulation of gene expression even where there is about 5%, 10%, 15% or 20% or more mis-match between the sequence used and the target gene. Effectively, the homology should be sufficient for the down-
40 regulation of gene expression to take place.

A suppressor RNA molecule may comprise 10-40 nucleotides of the sense or anti-sense strand of a nucleic acid sequence which encodes DA2, DA1 and/or EOD1 polypeptide.

5 Suppressor nucleic acids may be operably linked to heterologous promoters, for example tissue-specific or inducible promoters. For example, integument and seed specific promoters can be used to specifically down-regulate two or more DA1, DA2 and/or EOD1 nucleic acids in developing ovules and seeds to increase final seed size.

10 In some preferred embodiments, DA2 suppressor nucleic acid may be expressed in a plant cell with a nucleic acid encoding a dominant negative DA1 polypeptide and optionally an EOD1 suppressor nucleic acid.

15 Nucleic acid encoding the suppressor nucleic acid and/or a dominant-negative DA1 polypeptide may be comprised in one or more vectors.

20 Nucleic acid encoding the suppressor nucleic acid(s) as described herein and/or dominant-negative DA1 polypeptide may be operably linked to a heterologous regulatory sequence, such as a promoter, for example a constitutive, inducible, tissue-specific or developmental specific promoter as described above.

25 Nucleic acid encoding suppressor nucleic acid(s) as described herein and/or dominant negative DA1 polypeptides may be contained on a nucleic acid construct or vector. The construct or vector is preferably suitable for transformation into and/or expression within a plant cell. A vector is, *inter alia*, any plasmid, cosmid, phage or
30 *Agrobacterium* binary vector in double or single stranded linear or circular form, which may or may not be self-transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host, in particular a plant host, either by integration into the cellular genome or exist extrachromasomally (e.g. autonomous replicating
35 plasmid with an origin of replication).

Specifically included are shuttle vectors by which is meant a DNA vehicle capable, naturally or by design, of replication in two different organisms, which may be selected from *Actinomyces* and
40 related species, bacteria and eukaryotic (e.g. higher plant, mammalia, yeast or fungal) cells.

A construct or vector comprising nucleic acid as described above need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Constructs and vectors may further comprise selectable genetic markers consisting of genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones, glyphosate and d-amino acids.

Those skilled in the art can construct vectors and design protocols for recombinant gene expression, for example in a microbial or plant cell. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 3rd edition, Sambrook et al, 2001, Cold Spring Harbor Laboratory Press and *Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds. John Wiley & Sons, 1992. Specific procedures and vectors previously used with wide success upon plants are described by Bevan, Nucl. Acids Res. (1984) 12, 8711-8721), and Guerinneau and Mullineaux, (1993) Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148.

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct that contains effective regulatory elements that will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, the target cell type is preferably such that cells can be regenerated into whole plants.

It is desirable to use a construct and transformation method which enhances expression of the nucleic acid encoding the suppressor nucleic acid or dominant negative DA1 polypeptide. Integration of a single copy of the gene into the genome of the plant cell may be beneficial to minimize gene silencing effects. Likewise, control of

the complexity of integration may be beneficial in this regard. Of particular interest in this regard is transformation of plant cells utilizing a minimal gene expression construct according to, for example, EP Patent No. EP1407000B1, herein incorporated by reference for this purpose.

Techniques well known to those skilled in the art may be used to introduce nucleic acid constructs and vectors into plant cells to produce transgenic plants with the properties described herein.

Agrobacterium transformation is one method widely used by those skilled in the art to transform plant species. Production of stable, fertile transgenic plants is now routine in the art (see for example Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, et al. (1988) *Plant Cell Rep.* 7, 379-384; Zhang, et al. (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, et al. (1989) *Nature* 338, 274-276; Datta, et al. (1990) *Bio/Technology* 8, 736-740; Christou, et al. (1991) *Bio/Technology* 9, 957-962; Peng, et al. (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao, et al. (1992) *Plant Cell Rep.* 11, 585-591; Li, et al. (1993) *Plant Cell Rep.* 12, 250-255; Rathore, et al. (1993) *Plant Molecular Biology* 21, 871-884; Fromm, et al. (1990) *Bio/Technology* 8, 833-839; Gordon-Kamm, et al. (1990) *Plant Cell* 2, 603-618; D'Halluin, et al. (1992) *Plant Cell* 4, 1495-1505; Walters, et al. (1992) *Plant Molecular Biology* 18, 189-200; Koziel, et al. (1993) *Biotechnology* 11, 194-200; Vasil, I. K. (1994) *Plant Molecular Biology* 25, 925-937; Weeks, et al. (1993) *Plant Physiology* 102, 1077-1084; Somers, et al. (1992) *Bio/Technology* 10, 1589-1594; WO92/14828; Nilsson, O. et al (1992) *Transgenic Research* 1, 209-220).

Other methods, such as microprojectile or particle bombardment (US 5100792, EP-A-444882, EP-A-434616), electroporation (EP 290395, WO 8706614), microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) *Plant Tissue and Cell Culture*, Academic Press), direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. *Plant Cell Physiol.* 29: 1353 (1984)) or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d)) may be preferred where Agrobacterium transformation is inefficient or ineffective, for example in some gymnosperm species. Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g. bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

Following transformation, a plant cell with reduced DA2 expression and reduced DA1 and/or EOD1 expression or activity may be identified and/or selected. A plant may be regenerated from the plant cell.

A plant with reduced DA2 activity or expression that is also deficient in the expression or activity of DA1, EOD1 or both DA1 and EOD1, as described above may be sexually or asexually propagated or grown to produce off-spring or descendants. Off-spring or descendants of the plant regenerated from the one or more cells may be sexually or asexually propagated or grown. The plant or its off-spring or descendants may be crossed with other plants or with itself.

The DA1, DA2 and/or EOD1 amino acid or nucleic acid sequence may be employed as a molecular marker to determine the expression or activity of one or more of the DA1, DA2 and/or EOD1 polypeptides in a plant before, during or after growing or sexually or asexually propagated as set out above. A method may comprise:

providing a population of plants,

determining the amount of expression of an DA1, DA2 and/or EOD1 polypeptide in one or more plants in the population, and
identifying one or more plants in the population with
reduced expression of the DA1, DA2 and/or EOD1 polypeptide
relative to other members of said population.

The population of plants may be produced as described above.

In some embodiments, a method may comprise:

crossing a first and a second plant to produce a population of progeny plants;

determining the expression of one or more of DA1, DA2 and EOD1 polypeptides in the progeny plants in the population, and

identifying a progeny plant in the population in which
expression of the DA1, DA2 and/or EOD1 polypeptide is reduced
relative to controls.

One or both of the first and second plants may be produced as described above.

A progeny plant in which expression of the DA2 and DA1 and/or EOD1 polypeptide is reduced relative to controls (e.g. other members of the population) may display increased seed and/or organ size relative to the controls and may have higher plant yields.

In some embodiments, DA1 and EOD1 amino acid or nucleic acid sequences may be employed as a molecular marker to determine the expression or activity of one or more of the DA1 and/or EOD1 polypeptides in a plant in order to identify a plant or plant cell deficient in DA1 and/or EOD1 in which expression or activity of a DA2 polypeptide may be reduced as described above. A method may comprise:

providing a population of plants,
determining the amount of expression of an DA1 and/or EOD1 polypeptide in one or more plants in the population, and
identifying one or more plants in the population with
reduced expression of the DA1 and/or EOD1 polypeptide relative to other members of said population.

DA2 expression or activity may be reduced in the identified plants as methods described above.

A plant or progeny plant may be identified by i) measuring the amount of DA1, DA2 and/or EOD1 polypeptide in one or more cells of the plant ii) measuring the amount of DA1, DA2 and/or EOD1 mRNA in one or more cells of the plant or iii) sequencing the nucleic acid encoding the DA1, DA2 and/or EOD1 polypeptide in one or more cells of the plant and identifying the presence of one or more mutations.

The identified plants may be further propagated or crossed, for example, with other plants having reduced DA1, DA2 and/or EOD1 expression or self-crossed to produce inbred lines. The expression or activity of a DA1, DA2 and/or EOD1 polypeptide in populations of progeny plants may be determined and one or more progeny plants with reduced expression or activity of DA1, DA2 and/or EOD1 identified.

In some embodiments, the amount of expression of DA1, DA2 and/or EOD1 may be determined at the protein level. A method may comprise:

- providing a population of plants,
- determining the amount of DA1, DA2 and/or EOD1 polypeptide in one or more plants of said population, and
- identifying one or more plants in the population with reduced amount of an DA1, DA2 and/or EOD1 polypeptide relative to other members of said population.

Conveniently, immunological techniques, such as Western blotting, may be employed, using antibodies which bind to the DA1, DA2 or EOD1 polypeptide and show little or no binding to other antigens in the plant. For example, the amount of an DA1, DA2 and/or EOD1 polypeptide in a plant cell may be determined by contacting a sample comprising the plant cell with an antibody or other specific binding member directed against the DA1, DA2 or EOD1 polypeptide, and determining binding of the DA1, DA2 or EOD1 polypeptide to the sample. The amount of binding of the specific binding member is indicative of the amount of DA1, DA2 or EOD1 polypeptide which is expressed in the cell.

The amount of DA1, DA2 and/or EOD1 polypeptide may be determined in one or more cells of the plant, preferably cells from an above-ground portion or tissue of the plant, such as the vasculature and primary and secondary meristems in the shoot.

In other embodiments, the expression of the DA1, DA2 or EOD1 polypeptide may be determined at the nucleic acid level. For example, the amount of nucleic acid encoding a DA1, DA2 or EOD1

polypeptide may be determined. A method of producing a plant having increased yield related traits may comprise:

providing a population of plants,

determining the level or amount of nucleic acid, for example mRNA, encoding the DA1, DA2 or EOD1 polypeptide in a cell of one or more plants of said population, and,

identifying one or more plants in the population with reduced amount of an DA1, DA2 or EOD1 encoding nucleic acid relative to other members of said population.

The level or amount of encoding nucleic acid in a plant cell may be determined for example by detecting the amount of transcribed encoding nucleic acid in the cell. This may be performed using standard techniques such as Northern blotting or RT-PCR.

Alternatively, the presence of sequence variations which affect the expression or activity of a DA1, DA2 or EOD1 polypeptide may be determined. Another method of producing a plant having increased growth and/or biomass may comprise:

providing a population of plants,

determining the presence of one or more sequence variations, for example, polymorphisms, mutations or regions of hypermethylation, in a nucleic acid encoding an DA1, DA2 and/or EOD1 polypeptide in a cell in one or more plants of said population,

wherein said one or more sequence variations which reduce the expression or activity of the encoded DA1, DA2 and/or EOD1 polypeptide, and

identifying one or more plants in the population with one or more sequence variations which reduce the expression or activity of DA1, DA2 and/or EOD1 relative to other members of said population.

DA1, DA2 and/or EOD1 polypeptides and encoding nucleic acid are described in more detail above.

The presence of one or more sequence variations in a nucleic acid may be determined by detecting the presence of the variant nucleic acid sequence in one or more plant cells or by detecting the presence of the variant polypeptide which is encoded by the nucleic acid sequence. Preferred nucleic acid sequence variation detection techniques include ARMS[™]-allele specific amplification, OLA, ALEX[™], COPS, Taqman, Molecular Beacons, RFLP, and restriction site based PCR and FRET techniques.

Numerous suitable methods for determining the amount of a nucleic acid encoding an DA1, DA2 or EOD1 polypeptide, or the presence or absence of sequence variation in a nucleic acid encoding an DA1, DA2 or EOD1 polypeptide, in a plant cell, are available in the art (see for example (see for example Molecular Cloning: a Laboratory Manual: 3rd edition, Sambrook & Russell (2001) Cold Spring Harbor Laboratory Press NY; Current Protocols in Molecular Biology, Ausubel et al. eds. John Wiley & Sons (1992); DNA Cloning, The Practical Approach Series (1995), series eds. D. Rickwood and B.D. Hames, IRL Press, Oxford, UK and PCR Protocols: A Guide to Methods and Applications (Innis, et al. 1990. Academic Press, San Diego, Calif.)). Many current methods for the detection of sequence variation are reviewed by Nollau et al., Clin. Chem. 43, 1114-1120, 1997; and in standard textbooks, for example "Laboratory Protocols for Mutation Detection", Ed. by U. Landegren, Oxford University Press, 1996 and "PCR", 2nd Edition by Newton & Graham, BIOS Scientific Publishers Limited, 1997.

Preferred polypeptide sequence variation techniques include immunoassays, which are well known in the art e.g. A Practical Guide to ELISA by D M Kemeny, Pergamon Press 1991; Principles and Practice of Immunoassay, 2nd edition, C P Price & D J Newman, 1997, published by Stockton Press in USA & Canada and by Macmillan Reference in the United Kingdom.

In some embodiments, nucleic acid or an amplified region thereof may be sequenced to identify or determine the presence of polymorphism or mutation therein. A polymorphism or mutation may be identified by comparing the sequence obtained with the known sequence of DA1, DA2 or EOD1, for example as set out in sequence databases. Alternatively, it can be compared to the sequence of the corresponding nucleic acid from control cells. In particular, the presence of one or more polymorphisms or mutations that cause reduction but not total abrogation of function may be determined. Sequencing may be performed using any one of a range of standard techniques. Sequencing of an amplified product may, for example, involve precipitation with isopropanol, resuspension and sequencing using a TaqFS+ Dye terminator sequencing kit (e.g. from GE Healthcare UK Ltd UK). Extension products may be electrophoresed on an ABI 377 DNA sequencer and data analysed using Sequence Navigator software.

A progeny plant identified as having reduced DA1, DA2 and/or EOD1 expression may be tested for increased or enhanced yield related traits, such as increased seed or organ size, relative to controls.

- 5 The identified progeny plant may be further propagated or crossed, for example with the first or second plant (i.e. backcrossing) or self-crossed to produce inbred lines.

10 The identified progeny plant may be tested for seed size, organ size and/or plant yield relative to controls.

A plant produced as described herein may be deficient in DA2 expression or activity and may be further deficient in DA1 expression or activity, EOD1 expression or activity or both DA1 and
15 EOD1 expression or activity.

The expression or activity of DA2, DA1 and EOD1 may be reduced or abolished in the plant by mutation or one or more nucleotides in the plant coding sequence and/or by the expression of a heterologous
20 nucleic acid encoding a suppressor nucleic acid. In some preferred embodiments, the activity of DA1 may be reduced or abolished in the plant by expression of a heterologous nucleic acid encoding a dominant-negative DA1 polypeptide.

25 A plant may thus comprise heterologous nucleic acid which encodes a suppressor nucleic acid, such as an siRNA or shRNA, which reduces the expression of one or more of DA1, DA2 and EOD1 or which encodes a dominant negative DA1 polypeptide.

30 Any combination of mutations, suppressor nucleic acids may be employed in a plant as described herein. For example, a plant may comprise i) a mutation which reduces DA2 activity or expression, a heterologous nucleic acid encoding a suppressor nucleic acid which reduces EOD1 expression and a heterologous nucleic acid encoding a
35 nucleic acid which encodes a dominant-negative DA1 polypeptide; ii) a heterologous nucleic acid encoding a suppressor nucleic acid which reduces DA2 expression or expression, a mutation which reduces EOD1 expression and a heterologous nucleic acid encoding a nucleic acid which encodes a dominant-negative DA1 polypeptide iii) heterologous
40 nucleic acids encoding suppressor nucleic acids which reduce EOD1 and DA2 expression and a heterologous nucleic acid encoding a nucleic acid which encodes a dominant-negative DA1 polypeptide, or iv) mutation which reduce EOD1 and DA2 activity or expression and a

heterologous nucleic acid encoding a nucleic acid which encodes a dominant-negative DA1 polypeptide.

5 In other embodiments, a plant may comprise i) a mutation which reduces DA2 activity or expression, a heterologous nucleic acid encoding a suppressor nucleic acid which reduces DA1 expression ii) a heterologous nucleic acid encoding a suppressor nucleic acid which reduces DA2 expression or expression, a mutation which reduces DA1 expression iii) heterologous nucleic acids encoding suppressor
10 nucleic acids which reduce DA1 and DA2 expression, iv) mutations which reduce DA1 and DA2 activity or expression or v) a mutation which reduces DA2 activity or expression or a heterologous nucleic acid encoding a suppressor nucleic acid which reduces DA2 expression and a heterologous nucleic acid encoding a nucleic acid which
15 encodes a dominant negative DA1 polypeptide.

Heterologous nucleic acids encoding the dominant-negative DA1 polypeptide and/or suppressor nucleic acids may be on the same or different expression vectors and may be incorporated into the plant
20 cell by conventional techniques.

Examples of suitable plants for use in accordance with any aspect of the invention described herein include monocotyledonous and dicotyledonous higher plant, for example an agricultural or crop
25 plant, such as a plant selected from the group consisting of *Lithospermum erythrorhizon*, *Taxus* spp, tobacco, cucurbits, carrot, vegetable brassica, melons, capsicums, grape vines, lettuce, strawberry, oilseed brassica, sugar beet, wheat, barley, maize, rice, soyabeans, peas, sorghum, sunflower, tomato, potato, pepper,
30 chrysanthemum, carnation, linseed, hemp and rye.

A plant produced as described above may be sexually or asexually propagated or grown to produce off-spring or descendants. Off-spring or descendants of the plant regenerated from the one or more
35 cells may be sexually or asexually propagated or grown. The plant or its off-spring or descendants may be crossed with other plants or with itself.

Another aspect of the invention provides a transgenic plant having
40 reduced or abolished expression or activity of DA2 polypeptide within one or more cells thereof, wherein the plant is deficient in the expression or activity of DA1, EOD1 or both DA1 and EOD1.

The plant may comprise an exogenous nucleic acid which reduces or abolishes the expression or activity of one or more of DA2, DA1 and EOD1. In some embodiments, the transgenic plant may express a dominant negative DA1 polypeptide that reduces the activity of DA1.

5

In some embodiments, the plant may have reduced or abolished expression of DA1, DA2 and EOD1 or may have reduced or abolished expression of DA2 and EOD1 and may express a dominant negative DA1.

10 In addition to a plant produced by a method described herein, the invention encompasses any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part or propagule of any of these, such as cuttings and seed, which may be used in reproduction or propagation, sexual or asexual. Also encompassed by the invention
15 is a plant which is a sexually or asexually propagated off-spring, clone or descendant of such a plant, or any part or propagule of said plant, off-spring, clone or descendant.

A suitable plant may be produced by a method described above.

20

The plant may have increased yield relative to control wild-type plants (i.e. identical plants in which the expression or activity of DA2 and optionally DA1 and/or EOD1 has not been reduced). For example, the mass of seeds (e.g. grain) or other plant product per
25 unit area may be increased relative to control plants.

For example, one or more yield-related traits in the plant may be improved. Yield-related traits may include life-span, organ size and seed size.

30

A yield related trait may be improved, increased or enhanced in the plant relative to control plants in which expression of the nucleic acid encoding the DA2 polypeptide is not abolished or reduced (i.e. identical plants in which the expression of DA2 and optionally DA1
35 and/or EOD1 has not been reduced or abolished).

A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to
40 Plant Breeders Rights.

DA1 is shown herein to physically interact with DA2 *in vivo*.
Compounds that disrupt or interfere with the interaction may be
useful in increasing seed or organ size and improving plant yield.

- 5 A method of identifying a compound that increase plant yield may
comprise;
determining the effect of a test compound on the binding of a
DA2 polypeptide to a DA1 polypeptide,
a reduction or abolition of binding being indicative that the
10 compound may be useful in increasing plant yield.

DA1 and DA2 polypeptides are described in more detail above.

- The DA1 and DA2 polypeptides may be isolated or may be expressed
15 recombinant or endogenously in a plant cell.

A compound that reduces or abolishes DA1/DA2 binding may be useful in
the treatment of plants to increase yield.

- 20 "and/or" where used herein is to be taken as specific disclosure of
each of the two specified features or components with or without the
other. For example "A and/or B" is to be taken as specific
disclosure of each of (i) A, (ii) B and (iii) A and B, just as if
each is set out individually herein.

- 25 Unless context dictates otherwise, the descriptions and definitions
of the features set out above are not limited to any particular
aspect or embodiment of the invention and apply equally to all
aspects and embodiments which are described.

- 30 Other aspects and embodiments of the invention provide the aspects
and embodiments described above with the term "comprising" replaced
by the term "consisting of" and the aspects and embodiments
described above with the term "comprising" replaced by the term
35 "consisting essentially of".

All documents mentioned in this specification are incorporated
herein by reference in their entirety for all purposes.

- 40 The contents of all database entries mentioned in this specification
are also incorporated herein by reference in their entirety for all

purposes. This includes the versions of any sequences which are current at the filing date of this application.

Experiments

1. Methods

1.1 Plant materials and growth conditions

Arabidopsis ecotype Columbia (Col-0) was the wild type line used. All mutants were in the Col-0 background. *da2-1* (SALK_150003) was obtained from Arabidopsis Stock Centre NASC and ABRC collections.

The T-DNA insertion was confirmed by PCR and sequencing. Seeds were surface-sterilized with 100% isopropanol for 1min and 10% (v/v) household bleach for 10min, washed at least three times with sterile water, stratified at 4°C for 3d in the dark, dispersed on GM medium with 0.9% agar and 1% glucose, and then grown at 22°C. Plants were grown under long-day condition (16h light/8h dark) at 22°C.

1.2 Constructs and transformation

The *pDA2:DA2* construct was made by using a PCR-based Gateway system. The 1960bp promoter sequence of *DA2* was amplified using the primers *DA2proGW-F* and *DA2proGW-R*. PCR products were then cloned to the *pCR8/GW/TOPO TA* cloning vector (Invitrogen). The *DA2* CDS was amplified and the PCR products were then cloned to the *AscI* and *KpnI* sites of the Gateway vector *pMDC110* to get the *DA2CDS-pMDC110* plasmid. The *DA2* promoter was then subcloned to the *DA2CDS-pMDC110* by LR reaction to generate the *pDA2:DA2* construct. The plasmid *pDA2:DA2* was introduced into the *da2-1* mutant plants using *Agrobacterium tumefaciens* GV3101 and transformants were selected on hygromycin (30µg/ml) containing medium.

The *35S:DA2* construct was made using a PCR-based Gateway system. PCR products were subcloned into the *pCR8/GW/TOPO TA* cloning vector (Invitrogen) using TOPO enzyme. The *DA2* gene was then subcloned into Gateway Binary Vector *pMDC32* containing the 35S promoter (Curtis and Grossniklaus, 2003). The plasmid *35S:DA2* was introduced into Col-0 plants using *Agrobacterium tumefaciens* GV3101 and transformants were selected on hygromycin (30µg/ml) -containing medium.

The 1960 bp promoter sequence of *DA2* was amplified and the PCR products were cloned to the *pGEM-T* vector (Promega) using T4 DNA ligase and sequenced. The *DA2* promoter was then inserted into the *SacI* and *NcoI* sites of the binary vector *pGreen-GUS* (Curtis and Grossniklaus, 2003) to generate the transformation plasmid *pDA2:GUS*. The plasmid *pDA2:GUS* was introduced into Col-0 plants using

Agrobacterium tumefaciens GV3101 and transformants were selected on kanamycin (50µg/ml) -containing medium. The 35S:GW2 construct was made using a PCR-based Gateway system. PCR products were subcloned into the pCR8/GW/TOPO TA cloning vector (invitrogen) using TOPO enzyme. The GW2 gene was then subcloned into Gateway Binary Vector pMDC32 containing the 35S promoter (Curtis and Grossniklaus, 2003). The plasmid 35S:GW2 was introduced into Col-0 plants using *Agrobacterium tumefaciens* GV3101 and transformants were selected on hygromycin (30µg/ml)-containing medium.

1.3 Morphological and cellular analysis

Average seed weight was determined by weighing mature dry seeds in batches of 500 using an electronic analytical balance (METTLER MOLEDO AL104 CHINA). The weights of five sample batches were measured for each seed lot. Seeds were photographed under a Leica microscope (LEICA S8APO) using a Leica CCD (DFC420) and seed size were measured by using Image J software. Area measurements of petals (stage 14), leaves, and cotyledons were made by scanning organs to produce a digital image, and then calculating area, length and width by using Image J software. Leaf, petal and embryo cell sizes were measured from DIC images. Biomass accumulation in flowers (stage 14) was measured by weighing organs.

1.4 GUS staining

Samples (pDA2:GUS) were stained in a solution of 1 mM X-gluc, 100 mM Na₃PO₄ buffer, 3mM each K₃Fe(CN)₆/K₄Fe(CN)₆, 10mM EDTA, and 0.1% Nodidet-P40, and incubated at room temperature for 6 hours. After GUS staining chlorophyll was removed using 70% ethanol.

1.5 RNA isolation, RT-PCR, and Quantitative real-time RT-PCR analysis

Total RNA was extracted from Arabidopsis roots, stems, leaves, seedlings and inflorescences using an RNeasy Plant Mini kit (TIANGEN, China). Reverse transcription (RT)-PCR was performed as described (Li et al., 2006). cDNA samples were standardized on actin transcript amount using the primers ACTIN2-F and ACTIN2-R. Quantitative real-time RT-PCR analysis was performed with a lightcycler 480 engine (Roche) using the lightcycler 480 SYBR Green Master (Roche). ACTIN7 mRNA was used as an internal control, and relative amounts of mRNA were calculated using the comparative threshold cycle method.

1.6 E3 ubiquitin ligase activity assay

The coding sequence of *DA2* was cloned into *BamH* I and *PstI* sites of the *pMAL-C2* vector to generate the construct *MBP-DA2*. The mutated *DA2* (*DA2C59S* and *DA2N91L*) were generated by following the instruction manual of multi-site directed mutagenesis kit (Stratagene).

Bacterial lysates expressing *MBP-DA2* and mutated *MBP-DA2* were prepared from *E. coli* BL21 induced with 0.4 mM IPTG for 2 hours. Bacteria were lysed in TGH lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail [Roche]) and sonicated. The lysates were cleared by centrifugation and incubated with amylose resin (New England Biolabs) at 4°C for 30 min. Beads were washed by column buffer (20 mM Tris pH7.4, 200 mM NaCl, 1 mM EDTA) and equilibrated by reaction buffer (50 mM Tris pH7.4, 20mM DTT, 5 mM MgCl₂, 2 mM ATP). 110 ng E1 (Boston Biochem), 170 ng E2 (Boston Biochem), 1 µg His-ubiquitin (Sigma-Aldrich), and 2 µg *DA2-MBP* or mutated *DA2-MBP* fusion protein was incubated in a 20µl reaction buffer for 2 hours at 30°C.

Polyubiquitinated proteins were detected by immunoblotting with an antibody against His (Abmart) and an antibody against *MBP* (New England Biolabs).

1.7 *In vitro* protein-protein interaction

The coding sequences of *DA1*, *dal-1*, and *DA1* derivatives containing specific protein domains were cloned into *BamH* I and *Not* I sites of the *pGEX-4T-1* vector to generated *GST-DA1*, *GST-DA1R358K*, *GST-DA1-UIM*, and *GST-DA1-LIM+C* constructs, and *EcoRI* and *XhoI* sites of the *pGEX-4T-1* vector to generate *GST-DA1-LIM* and *GST-DA1-C* constructs.

To test protein-protein interaction, bacterial lysates containing approximately 15 µg of *MBP-DA2* fusion proteins were combined with lysates containing approximately 30 µg of *GST-DA1*, *GST-DA1R358K*, *GST-DA1-UIM*, *GST-DA1-LIM*, *GST-DA1-LIM+C* or *GST-DA1-C* fusion proteins. 20 µl amylose resin (New England Biolabs) was added into each combined solution with continued rocking at 4°C for 1 hour. Beads were washed times with TGH buffer, and the isolated proteins were separated on a 10% SDS-polyacrylamide gel and detected by western blot analysis with anti-GST (Abmart) and anti-MBP antibodies (Abmart), respectively.

1.8 Co-immunoprecipitation

The coding sequence of *DA1* and *DA1-C* was cloned into *KpnI* and *BamHI* sites of the *pCAMBIA1300-221-Myc* vector to generate the transformation plasmid *35S::Myc-DA1* and *35S::Myc-DA1-C*. PCR products were subcloned into the *pCR8/GW/TOPO* TA cloning vector (Invitrogen) using TOPO enzyme. The *DA2* gene was then subcloned into Gateway Binary Vector *pMDC43* containing the 35S promoter and the *GFP* gene (Curtis and Grossniklaus, 2003). PCR products were subcloned into the *pCR8/GW/TOPO* TA cloning vector (Invitrogen) using TOPO enzyme. The *PEX10* gene were then subcloned into Gateway Binary Vector *pH7FWG2* containing the 35S promoter and the *GFP* gene.

Nicotiana benthamiana leaves were transformed by injection of *Agrobacterium tumefaciens* GV3101 cells harboring *35S::Myc-DA1* and *35S::GFP-DA2* plasmids as previously described (Voinnet et al., 2003). Total protein was extracted with extraction buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 20% glycerol, 2% Triton X-100, 1mM EDTA, 1×complete protease inhibitor cocktail (Roche) and MG132 20ug/ml) and incubated with GFP-Trap-A (Chromotek) for 1 hour at 4°C. Beads were washed 3 times with wash buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 1× complete protease inhibitor cocktail (Roche)). The immunoprecipitates were separated in 10% SDS-polyacrylamide gel and detected by western blot analysis with anti-GFP (Beyotime) and anti-Myc (Abmart) antibodies, respectively.

1.9 Accession numbers

Arabidopsis Genome Initiative locus identifiers for Arabidopsis genes mentioned herein are as follows: Atlg19270 (NP_173361.1 GI:15221983) (*DA1*), At4g36860 (NP_195404.6 GI:240256211) (*DAR1*), Atlg78420 (NP_001185425.1 GI:334183988) (*DA2*), Atlg17145 (NP_564016.1 GI:18394446) (*DA2L*), and At3g63530 (NP_001030922.1 GI:79316205) (*EOD1/BB*).

2. Results

2.1 The *da2-1* mutant produces large seeds

To further understand the mechanisms of ubiquitin-mediated control of seed size, we collected the publicly available T-DNA insertion lines of some predicted ubiquitin ligase genes that were expressed in Arabidopsis ovules and/or seeds in several microarray studies and investigated their seed growth phenotypes. From this screen, we identified several T-DNA insertion mutants with altered seed size. We designated one of these mutants *da2-1*, referring to the order of discovery for large seed size mutants (DA means "large" in Chinese). Seeds produced by *da2-1* were larger and heavier than the

wild-type seeds (Figures 1A, 3C and 3D). Seed number per silique and seed yield per plant in *da2-1* were slightly higher than those in wild type (Figures 1B and 1C). By contrast, total number of seeds per plant in *da2-1* was not significantly increased, compared with that in wild type (Figure 1D). The *da2-1* plants were higher than wild-type plants at the mature stage (Figure 1E). In addition, *da2-1* mutant plants formed large flowers and leaves as well as increased biomass compared with wild-type plants (Figure 2; Figure 15). The increased size of *da2-1* mutant petals and leaves was not caused by larger cells (Figure 15), indicating that it is the number of petal and leaf cells that is higher.

2.2 *DA2* acts synergistically with *DA1* to control seed size, but does so independently of *EOD1*

The *da2-1* mutant showed a weak but similar seed size phenotype to *dal-1* (Li et al., 2008), providing indication that *DA1* and *DA2* could function in a common pathway. To test for a genetic interaction between *DA1* and *DA2*, we generated a *dal-1 da2-1* double mutant and determined its seed size. Although the *da2-1* mutant had slightly larger and heavier seeds than wild type (Figures 1A, 3C and 3D), the *da2-1* mutation synergistically enhanced the seed size and weight phenotypes of *dal-1* (Figures 3A and 3C), revealing a synergistic genetic interaction between *DA1* and *DA2* in seed size. The changes in seed size were reflected in the size of the embryos and resulting seedlings (Figure 3B). We further measured cotyledon area of 10-d-old seedlings. A synergistic enhancement of cotyledon size of *dal-1* by the *da2-1* mutation was also observed (Figures 3B and 4). The mutant protein encoded by the *dal-1* allele has a negative activity toward *DA1* and a *DA1*-related protein (*DAR1*), the most closely-related family member (Li et al., 2008).

Double *dal-kol dar1-1* T-DNA insertion mutants exhibited the *dal-1* phenotypes, while *dal-kol* and *dar1-1* single mutants did not show an obvious seed size phenotype (Li et al., 2008). As *dal-1* and *da2-1* act synergistically to increase seed size, one would expect that the *dal-kol* might synergistically enhance the phenotypes of *da2-1*. To test this, we generated the *dal-kol da2-1* double mutant. As shown in Figure 3D, the seed size and weight phenotypes of *da2-1* were also synergistically enhanced by the *dal-kol* mutation. We further measured cotyledon area of 10-d-old seedlings. The *dal-kol* mutation synergistically enhanced the cotyledon size phenotype of *da2-1* (Figure 4 top right). Similarly, a synergistic enhancement of petal size of *da2-1* by the *dal-kol* mutation was also observed (Figure

16D). These results further demonstrate the synergistic effects of the simultaneous disruption of both *DA1* and *DA2*.

We further measured the size of embryo cells and petal epidermal cells. Cell size in *dal-1 da2-1* and *dal-ko1 da2-1* double mutants was not increased, compared with that measured in their parental lines (Figure 4 lower left; Figure 16E), providing indication that *DA1* and *DA2* act synergistically to restrict cell proliferation processes.

The *dal-1 da2-1* double mutant had larger seeds than *dal-ko1 da2-1* double mutants (Figures 3C, 3D and 4), which is consistent with our previous report that the *dal-1* allele had stronger phenotypes than *dal-ko1* (Li et al., 2008). The size of *dal-1* seeds was similar to that of *dal-ko1 dar1-1* double mutant seeds because the *dal-1* allele has a negative activity toward *DA1* and *DAR1* (Figure 4 lower right) (Li et al., 2008). Therefore, one would expect that the size of *dal-1 da2-1* double mutant seeds might be similar to that of *dal-ko1 dar1-1 da2-1* triple mutant seeds. We therefore generated a *dal-ko1 dar1-1 da2-1* triple mutant and investigated its seed size. As shown in Figure 4, the size of *dal-ko1 dar1-1 da2-1* triple mutant seeds was comparable with that of *dal-1 da2-1* double mutant seeds, but larger than that of *dal-ko1 da2-1* double mutant seeds. Thus, these genetic analyses further support that the *dal-1* allele has a negative effect on both *DA1* and *DAR1* (Li et al., 2008).

We have previously identified an enhancer of *dal-1* (*EOD1*), which is allelic to *BIG BROTHER* (*BB*) (Disch et al., 2006; Li et al., 2008). The *eod1* mutations synergistically enhanced the seed size phenotype of *dal-1* (Li et al., 2008). Similarly, the seed size and weight phenotypes of *da2-1* were synergistically enhanced by *dal-1* and *dal-ko1* (Figures 3A, 3C and 3D). We therefore asked whether *DA2* and *EOD1* could function in a common pathway. To determine genetic relationships between *DA2* and *EOD1*, we analyzed an *eod1-2 da2-1* double mutant. The genetic interaction between *eod1-2* and *da2-1* was essentially additive for both seed weight and petal size compared with their parental lines (Figure 16), providing indication that *DA2* functions to influence seed and organ growth separately from *EOD1*.

2.3 *DA2* acts maternally to influence seed size

Considering that the size of seeds is affected by the maternal and/or zygotic tissues, we asked whether *DA2* functions maternally or zygotically. To test this, we performed reciprocal cross experiments between wild type and *da2-1*. As shown in Figure 6, the effect of

da2-1 on seed size was observed only when maternal plants are homozygous for the *da2-1* mutation. Seeds produced by maternal *da2-1* plants, regardless of the genotype of the pollen donor, were consistently larger than those produced by maternal wild-type plants. This result indicates that *da2-1* can act maternally to influence seed size. We have previously demonstrated that *DA1* also functions maternally to control seed size (Li et al. 2008). As the *dal-kol* mutation synergistically enhanced the seed size phenotype of *da2-1* (Figure 3D), we further conducted reciprocal cross experiments between wild type and *dal-kol da2-1* double mutant. Similarly, the effect of *dal-kol da2-1* on seed size was observed only when *dal-kol da2-1* acted as the maternal plant (Figure 6).

Pollinating *dal-kol/+ da2-1/+* plants with *dal-kol da2-1* double mutant pollen leads to the development of *dal-kol da2-1*, *dal-kol/dal-kol da2-1/+*, *dal-kol/+ da2-1da2-1* and *dal-kol/+ da2-1/+* embryos within *dal-kol/+ da2-1/+* seed coats. We further measured the size of individual seeds from *dal-kol/+ da2-1/+* plants fertilized with *dal-kol da2-1* double mutant pollen and genotyped *dal-kol* and *da2-1* mutations. Our results show that *dal-kol* and *da2-1* mutations are not associated with variation in the size of these seeds (Figure 6). Together, these analyses indicate that the embryo and endosperm genotypes for *DA1* and *DA2* do not affect seed size, and *DA1* and *DA2* are required in sporophytic tissue of the mother plant to control seed growth.

2.4 *DA2* acts synergistically with *DA1* to affect cell proliferation in the maternal integuments

The reciprocal crosses showed that *DA1* and *DA2* function maternally to determine seed size (Figure 6) (Li et al., 2008). The integuments surrounding the ovule are maternal tissues and form the seed coat after fertilization, which may physically restrict seed growth. Several studies showed that the integument size of ovules determines seed size (Schruff et al., 2006; Adamski et al., 2009). We therefore asked whether *DA1* and *DA2* act through the maternal integuments to control seed size. To test this, we investigated mature ovules from wild type, *dal-1*, *da2-1* and *dal-1 da2-1* at 2 days after emasculation. The size of *dal-1* ovules was dramatically larger than that of wild-type ovules (Figures 5 and 7), consistent with our previous findings (Li et al., 2008).

The *da2-1* ovules were also larger than wild-type ovules (Figures 5, and 7). The *da2-1* mutation synergistically enhanced the ovule size

phenotype of *dal-1*, consistent with their synergistic interactions in seed size.

We investigated the outer integument cell number of developing seeds in wild type, *dal-1*, *da2-1* and *dal-1 da2-1* at 6 DAP and 8 DAP. In wild-type seeds, the number of outer integument cells at 6 DAP was similar to that at 8 DAP (Figure 7 middle panel), indicating that cells in the outer integuments of wild-type seeds completely stop division at 6 DAP. Similarly, cells in the outer integuments of *dal-1*, *da2-1* and *dal-1 da2-1* seeds completely stopped cell proliferation at 6 DAP. The number of outer integument cells in *dal-1* and *da2-1* seeds was significantly increased compared with that in wild-type seeds (Figure 7). The *da2-1* mutation synergistically enhanced the outer integument cell number of *dal-1*. We further investigated the outer integument cell length of wild-type, *dal-1*, *da2-1* and *dal-1 da2-1* seeds at 6 and 8 days after pollination. Cells in *dal-1*, *da2-1* and *dal-1 da2-1* outer integuments were significantly shorter than those in wild-type outer integuments (Figure 7 right panel), providing indication of a compensation mechanism between cell proliferation and cell expansion in the integuments. Thus, these results show that *DA2* acts synergistically with *DA1* to restrict cell proliferation in the maternal integuments.

2.5 *DA2* encodes a functional E3 ubiquitin ligase

The *da2-1* mutation was identified with T-DNA insertion in the seventh exon of the gene *Atlg78420* (Figure 8A). The T-DNA insertion site was further confirmed by PCR using T-DNA specific and flanking primers and sequencing PCR products. The full-length mRNA of *Atlg78420* could not be detected by semi-quantitative RT-PCR in *da2-1* mutant. We expressed the *Atlg78420* CDS under the control of its own promoter in *da2-1* plants and isolated 62 transgenic plants. Nearly all transgenic lines exhibited complementation of *da2-1* phenotypes (Figure 10), indicating that *Atlg78420* is the *DA2* gene.

To further characterize *DA2* function, in particular gain of function phenotypes, we expressed the coding region of *DA2* under the control of the CaMV 35S promoter in wild-type plants and isolated 77 transgenic plants. Overexpression of *DA2* caused decreases in seed size, seed yield per plant and seed number per plant (Figures 1A, 1C and 1D). In addition, most transgenic plants overexpressing *DA2* had small flowers and leaves, short siliques, reduced plant height as well as decreased biomass compared with wild type (Figures 1E, 2 and

15). These results further support the role of DA2 in limiting seed and organ growth.

The DA2 gene is predicted to encode a 402 -amino-acid protein containing one predicted RING domain (59-101) (Figure 8B; Table 1). To investigate whether DA2 has E3 ubiquitin ligase activity, we expressed DA2 in *Escherichia coli* as a fusion protein with maltose binding protein (MBP) and purified MBP-DA2 protein from the soluble fraction. In the presence of an E1 ubiquitin activating enzyme, an E2 conjugating enzyme, His-ubiquitin and MBP-DA2, a polyubiquitination signal was observed by western blot using an anti-His antibody (Figure 9, fifth lane from the left). The anti-MBP blot analysis also showed that MBP-DA2 was ubiquitinated (Figure 9, fifth lane from the left). However, in the absence of any of E1, E2, His-ubiquitin or MBP-DA2, no polyubiquitination was detected (Figure 9, first to fourth lanes from the left), demonstrating that DA2 is a functional E3 ubiquitin ligase. The RING motif is essential for the E3 ubiquitin ligase activity of RING finger proteins (Xie et al., 2002). Therefore, we tested whether an intact RING finger domain was required for DA2 E3 ligase activity. A single amino acid substitution allele was produced by mutagenizing Cysteine-59 to Serine (C59S), as this mutation is predicted to disrupt the RING domain (Tables 1 and 2). An *in vitro* ubiquitination assay indicated that the E3 ligase activity was abolished in the C59S mutant of DA2 (Figure 9, sixth lane from the left), indicating that an intact RING domain is required for DA2 E3 ubiquitin ligase activity. We further overexpressed DA2 C59S (35S:DA2C59S) in wild-type Col-0 plants and isolated 69 transgenic plants. The seed size of transgenic plants was comparable with that of wild-type plants although transgenic plants had high expression levels of DA2 C59S, indicating that the DA2 C59S mutation affects the function of DA2 in seed growth.

Three RING types, RING-H2, RING-HCa and RING-HCb, and five modified RING types, RING-C2, RING-v, RING-D, RING-S/T and RING-G have been described in *Arabidopsis* (Stone et al., 2005). A new type of RING domain (C5HC2) found in rice GW2 has been proposed (Song et al., 2007). Although the spacing of the cysteines in the predicted RING domain of DA2 was similar to that in the RING domain (C5HC2) of rice GW2, the RING domain of DA2 lacked a conserved histidine residue that was replaced by an asparagine residue (Asn-91) (Tables 1 and 2). This amino acid substitution was also observed in the predicted RING domain of DA2 homologs in dicots, such as soybean and oilseed rape (Table 1). We therefore asked whether this asparagine residue

(Asn-91) is crucial for its E3 ubiquitin ligase activity. A single amino acid substitution allele was produced by mutagenizing Asn-91 to Leucine (N91L). An *in vitro* ubiquitination assay showed that the N91L mutant of DA2 had the E3 ligase activity (Figure 9, the seventh lane from the left), suggesting that Asn-91 may not be required for DA2 E3 ligase activity. These results suggest that the RING domain of DA2 might be a variant of that found in GW2. We further overexpressed *DA2 N91L* (*35S:DA2N91L*) in wild-type plants and isolated 26 transgenic plants. The seeds of transgenic plants were smaller than wild-type seeds, suggesting that the *DA2 N91L* could restrict seed growth.

2.6 Homologs of Arabidopsis DA2

Proteins that share significant homology with DA2 outside of the RING domain are found in Arabidopsis and crop plants including oilseed rape, soybean, rice, maize and barley (Table 2). One predicted protein in Arabidopsis shares extensive amino acid similarity with DA2 and is named DA2-like protein (DA2L; At1g17145). Like *35S:DA2* plants, *DA2L*-overexpressing lines exhibited small plants and organs (Figure 18), providing indication that *DA2* and *DA2L* have similar functions. The similar proteins in other plant species show a 39.2%–84.5% amino acid sequence identity with DA2 (Table 2). The homolog in *Brassica napus* had the highest amino acid sequence identity with DA2 (84.5%) (Table 2). Rice GW2 had 43.1% amino acid sequence identities with Arabidopsis DA2 (Table 2). As overexpression of *GW2* reduced grain width in rice (Song et al., 2007), we asked whether *DA2* and *GW2* performs similar function in seed size control. We therefore overexpressed *GW2* in wild-type plants. Like *35S:DA2* and *35S:DA2L* transgenic lines, the Arabidopsis transgenic plants overexpressing *GW2* produced smaller seeds and organs than wild-type plants, indicating conserved function for Arabidopsis *DA2* and rice *GW2* in seed and organ growth control.

2.7 *DA2* and *DA1* show similar expression patterns

To determine the expression pattern of *DA2*, RNAs from roots, stems, leaves, seedlings and inflorescences were analyzed by quantitative real-time RT-PCR analysis. *DA2* mRNA was detected in all plant organs tested (Figure 11A). The tissue-specific expression patterns of *DA2* were investigated using histochemical assay of GUS activity of transgenic plants containing a *DA2* promoter:*GUS* fusion (*pDA2:GUS*). GUS activity was detected in roots, cotyledons, leaves and inflorescences (Figures 11B and 11C). Relatively high GUS activity was detected in leaf primordia and roots (Figures 11B and 11C). In

flowers, relatively stronger expression of *DA2* was observed in young floral organs than old floral organs (Figures 11D-11L). Similarly, higher GUS activity was detected in younger ovules than older ones (Figures 11M and 11N). This shows that *DA2* expression is regulated temporally and spatially.

2.8 DA1 interacts with DA2 *in vitro* and *in vivo*

Our genetic analyses show that DA1 acts synergistically with DA2 to restrict seed and organ growth. We therefore assessed whether DA1 interacts with the E3 ubiquitin ligase DA2 using an *in vitro* interaction/pull-down experiment. DA1 was expressed as a GST fusion protein, while DA2 was expressed as a MBP fusion protein. As shown in Figure 12 (first and second lanes from left), GST-DA1 bound to MBP-DA2, while GST-DA1 did not bind to a negative control (MBP). This result indicates that DA1 physically interacts with DA2 *in vitro*.

DA1 contains two ubiquitin interaction motifs (UIM), a single LIM domain and the highly conserved C-terminal region (Figure 13) (Li et al., 2008). We further asked which domain of DA1 is required for interaction between DA1 and DA2. A series of DA1 derivatives containing specific protein domains were expressed in *Escherichia coli*: DA1-UIM containing only the two UIM domains, DA1-LIM with only the LIM domain, DA1-LIM+C containing only the LIM domain and the C-terminal region, and DA1-C with only the C-terminal region, were expressed as GST fusion proteins (Figure 13).

DA2 was expressed as an MBP fusion protein and used in pull-down experiments. As shown in Figure 12, GST-DA1-LIM+C and GST-DA1-C interacted with MBP-DA2, but the GST-DA1-UIM and GST-DA1-LIM did not bind to MBP-DA2. This result indicates that the conserved C-terminal region of DA1 interacts with DA2.

Considering that the mutant protein encoded by the *dal-1* allele (DA1R358K) has a mutation in the C-terminal region (Figure 13) (Li et al., 2008), we asked whether the DA1R358K mutation affects interactions with DA2. Using a GST-DA1R358K fusion protein in pull-down experiments with MBP-DA2, we showed that the mutation in DA1R358K does not affect the interaction between DA1 and DA2 (Figure 12, third lane from left).

To further investigate possible association between DA1 and DA2 in planta, we used coimmunoprecipitation analysis to detect their

interactions *in vivo*. We transiently coexpressed 35S:Myc-DA1 and 35S:GFP-DA2 in *Nicotiana benthamiana* leaves. Transient coexpression of 35S:GFP and 35S:Myc-DA1 in *Nicotiana benthamiana* leaves was used as a negative control. Total proteins were isolated and incubated with GFP-Trap-A agarose beads to immunoprecipitate GFP-DA2 or GFP. Precipitates were detected with anti-GFP and anti-Myc antibodies, respectively. As shown in Figure 14, Myc-DA1 was detected in the immunoprecipitated GFP-DA2 complex but not in the negative control (GFP), indicating that there is a physical association between DA1 and DA2 in planta. As the C-terminal region of DA1 interacted with DA2 in the pull-down assay (Figure 12), we further asked whether the C-terminus of DA1 interacts with DA2 in planta. The co-immunoprecipitation analysis showed that the C-terminal region of DA1 (Myc-DA1-C) was detected in the GFP-DA2 complex but not in the negative control (PEX10-GFP, a RING-type E3 ubiquitin ligase) (Platta et al., 2009; Kaur et al., 2013). Thus, these results indicate that the C-terminal region of DA1 is required for interaction with DA2 *in vitro* and *in vivo*.

Seed size in higher plants is a key determinant of evolutionary fitness, and is also an important agronomic trait in crop domestication (Gomez, 2004; Orsi and Tanksley, 2009). Several factors that act maternally to control seed size have been identified, such as ARF2/MNT, AP2, KLU/CYP78A5, EOD3/CYP78A6 and DA1. However, the genetic and molecular mechanisms of these factors in seed size control are nearly totally unknown. We previously demonstrated that the ubiquitin receptor DA1 acts synergistically with the E3 ubiquitin ligase EOD1/BB to control seed size (Li et al., 2008).

In this study, we identified Arabidopsis DA2 as another RING E3 ubiquitin ligase involved in controlling seed size. Genetic analyses show that DA2 functions synergistically with DA1 to control final seed size, but does so independently of the E3 ubiquitin ligase EOD1. We further revealed that DA1 interacts physically with DA2. Our results define a ubiquitin-based system involving DA1, DA2 and EOD1 that controls final seed size in Arabidopsis.

2.9 DA2 acts maternally to control seed size

The *da2-1* loss-of-function mutant formed large seeds and organs, whereas plants overexpressing DA2 produced small seeds and organs (Figure 1A), indicating that DA2 is a negative factor of seed and organ size control. Surprisingly, Arabidopsis DA2 has been recently

proposed as a positive regulator of organ growth, although nothing is known about how DA2 controls seed and organ growth (Van Daele et al., 2012). In this study, we have sufficient evidence to prove that DA2 acts as a negative factor of seed and organ growth control. The *da2-1* loss-of-function mutant formed large seeds and organs (Figures 1 to 4). Supporting this, the *da2-1* mutation synergistically enhanced the seed and organ size phenotypes of *dal-1* and *dal-kol* (Figures 1 to 4). The *da2-1* mutation also enhanced the seed and organ size phenotypes of *eod1-2*, further indicating that the *da2-1* mutation promotes seed and organ growth. The *da2-1* mutant formed large ovules with more cells in the integuments, and the *da2-1* mutation synergistically enhanced the ovule size phenotype of *dal-1* (Figure 6).

In addition, most transgenic plants overexpressing DA2 and DA2L were smaller than wild-type plants (Figure 2; Figure S9). The organ growth phenotypes of these transgenic plants were correlated with their respective expression levels (Figures S4 and S9). Therefore, our data clearly demonstrate that DA2 functions as a negative regulator of seed and organ size. Several Arabidopsis mutants with large organs also formed large seeds (Krizek, 1999; Mizukami and Fischer, 2000; Schruff et al., 2006; Li et al., 2008; Adamski et al., 2009), suggesting a possible link between organ size and seed growth. By contrast, several other mutants with large organs exhibited normal sized seeds (Hu et al., 2003; White, 2006; Xu and Li, 2011), indicating that organ and seed size is not invariably positively related. These results suggest that seeds and organs have both common and distinct pathways to control their respective size.

Reciprocal cross experiments showed that DA2 acts maternally to influence seed growth, and the embryo and endosperm genotypes for DA2 do not affect seed size (Figures 6). The integuments surrounding the ovule are maternal tissues and form the seed coat after fertilization. Alterations in maternal integument size, such as those seen in *arf2*, *dal-1* and *klu* ovules, have been known to contribute to changes in seed size (Schruff et al., 2006; Li et al., 2008; Adamski et al., 2009). Mature *da2-1* ovules were larger than mature wild-type ovules (Figures 5 and 7). The *da2-1* mutation also synergistically enhanced the integument size of *dal-1* ovules. Thus, a general theme emerging from these studies is that the control of maternal integument size is one of key mechanisms for determining final seed size. Consistent with this notion, plant maternal factors

of seed size control (e.g. KLU, ARF2 and DA1) isolated to date influence integument size (Schruff et al., 2006; Li et al., 2008; Adamski et al., 2009).

5 The size of the integument or seed coat is determined by cell proliferation and cell expansion, two processes that are coordinated. Cell number in the integuments of the mature ovule sets the growth potential of the seed coat after fertilization. For example, *arf2* mutants produced large ovules with more cells, leading
10 to large seeds (Schruff et al. 2006), while *klu* mutants had small ovules with less cells, resulting in small seeds (Adamski et al., 2009). Our results show that the integuments of *da1-1* and *da2-1* seeds had more cells than those of wild-type seeds, and *da1-1* and *da2-1* acts synergistically to promote cell proliferation in the
15 integuments. We also observed that cells in the outer integuments of *da1-1*, *da2-1*, and *da1-1 da2-1* seeds were shorter than those in wild-type integuments, suggesting that a possible compensation mechanism between cell proliferation and cell elongation in the maternal integument. Thus, it is possible that the maternal integument or
20 seed coat, which acts as a physical constraint on seed growth, can set an upper limit to final seed size.

2.10 A genetic framework for ubiquitin-mediated control of seed size

25 DA2 encodes a protein with one predicted RING domain that is distinctive from any of the previously described plant RING domains. The RING domain of DA2 shared highest homology with that of rice GW2 (C5HC2), but it lacked one conserved metal ligand amino acid (a histidine residue) that was replaced by an asparagine residue (Song
30 et al., 2007). It is still possible that the RING domain of DA2 might be a variant of that found in GW2. Many RING-type domains are found in E3 ubiquitin ligases that ubiquitinate substrates, often targeting them for subsequent proteasomal degradation (Smalle and Vierstra, 2004). We tested the E3 activity of recombinant DA2 in an
35 *in vitro* ubiquitin-ligase assay and demonstrated that DA2 is a functional E3 ubiquitin ligase, suggesting that DA2 may target positive regulators of cell proliferation for ubiquitin-dependent degradation by the 26S proteasome. Proteins that share homology with DA2 outside the RING domain are found in Arabidopsis and other plant
40 species. In Arabidopsis, the DA2-like protein (DA2L) shares extensive amino acid similarity with DA2. Like 35S:DA2 plants, DA2L-overexpressing lines showed small plants (Figure 18),

indicating that *DA2* and *DA2L* may perform similar functions. The homolog of *DA2* in rice is the RING-type (C5HC2) protein *GW2* (Song et al., 2007), which has been known to act as a negative regulator of seed size. However, the genetic and molecular mechanisms of *GW2* in seed size control are largely unknown in rice.

We previously identified *DA1*, a ubiquitin receptor with ubiquitin-binding activity, as a negative regulator of seed size (Li et al., 2008). A modifier screen identified an enhancer of *dal-1* (*EOD1*) (Li et al., 2008), which is allelic to the E3 ubiquitin ligase *BB* (Disch et al., 2006). Analysis of double *eod1-2 dal-1* mutants revealed synergistic genetic interactions between *DA1* and *EOD1* (Li et al., 2008), suggesting they may control seed growth by modulating the activity of a common target(s). Although genetic interactions between *dal-1* and *eod1-2* also synergistically enhanced seed and organ size, our genetic analyses show that *DA2* acts independently of *EOD1* to influence seed growth, suggesting *DA2* and *EOD1* may target distinct growth stimulators for degradation, with common regulation via *DA1*. Thus, our findings establish a framework for the control of seed and organ size by three ubiquitin-related proteins *DA1*, *DA2* and *EOD1*. In addition, we observed that overexpression of *GW2* restricts seed and organ growth in *Arabidopsis*, providing indication of a possible conserved function in *Arabidopsis* and rice. It could be interesting to investigate the effects of the combination of *GW2* and rice homologs of *DA1* and *EOD1* on grain size in rice.

2.11 A possible molecular mechanism of *DA1* and *DA2* in seed size control

Our results demonstrate that the E3 ubiquitin ligase *DA2* interacts with the ubiquitin receptor *DA1* *in vitro* and *in vivo* (Figures 12-14). However, it is not likely that *DA2* targets *DA1* for proteasomal degradation because a T-DNA inserted mutant of the *DA1* gene (*dal-kol*) synergistically enhances the seed size phenotype of *da2-1* (Figures 3 and 4). Nevertheless, many other types of ubiquitin modification regulate proteins in a proteasome independent manner (Schnell and Hicke, 2003). For example, monoubiquitination has been implicated in the activation of signaling proteins, endocytosis, and histone modification (Schnell and Hicke, 2003). In animals, monoubiquitination of the ubiquitin receptor *eps15* depends on interaction between *eps15* and the *Nedd4* family of E3 ligases (Woelk et al., 2006). In contrast, an E3-independent monoubiquitination of ubiquitin receptors has also been reported (Hoeller et al., 2007). Considering that *DA1* interacts with *DA2*, we tested whether *DA2* can

ubiquitinate or monoubiquitinate DA1. In the presence of E1, E2 and ubiquitin, DA2-His had an E3 ubiquitin ligase activity. However, in the presence of E1, E2, ubiquitin and DA2-His (E3), no ubiquitinated DA1-HA was detected under our reaction conditions. Ubiquitin

5 receptors can interact with polyubiquitinated substrates of E3s via UIM domains and facilitate their degradation by the proteasome (Verma et al., 2004). We previously demonstrated that UIM domains of DA1 can bind ubiquitin (Li et al., 2008).

10 Taken together with its interaction with DA2 through its C-terminal region (Figures 12 and 14), DA1 may be involved in mediating the degradation of the ubiquitinated substrates of DA2 by the proteasome. One mechanism may involve DA1 interaction with DA2, which helps DA1 specifically recognize the ubiquitinated
15 substrate(s) of DA2. DA1 may subsequently bind polyubiquitin chains of the ubiquitinated substrate(s) through its UIM domain and mediate the degradation of the ubiquitinated substrate(s). Improving seed yield is an important target for crop breeders worldwide, and the size of seeds is an important component of overall seed yields. We
20 identified DA2 as an important regulator of seed size that functions synergistically with DA1 to influence seed size.

DA1 also acts synergistically with EOD1 to affect seed growth. Overexpression of a dominant negative *dal-1* mutation (*Zmdal-1*) has
25 been reported to increase seed mass of corn (Wang et al., 2012), indicating the promise of combining the effects of DA1, DA2 and EOD1 from different seed crops to engineer large seed size in these crops.

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Bd_Bradi3g09270	CPICFLYYPSLN	SSCCS	GICTECFLQM	PTHTA	PTQCPFC
Hv_Yrg1	CPICFLYYPSLN	SSCCS	GICTECFLQM	PTHTA	PTQCPFC
Zm_gi 220961719	CPICFLYYPSLN	SSCCS	GICTECFLQM	PTHTA	PTQCPFC
Sb_gi 242064618	CPICFLYYPSLN	SSCCS	GICTECFLQM	PTHTA	PTQCPFC
Os_GW2	CPICFLYYPSLN	SSCCS	GICTECFLQM	PTHTA	PTQCPFC
Pt_gi 224061326	CPICFLYYPSLN	SSCCM	GICTECFLQM	NPNST	PTQCPFC
Cp_evm.model.supercontig_77	CPICFLYYPSLN	SSCCM	GICTECFLQM	NPNST	PTQCPFC
Rc_gi 255578534	CPICFLYYPSLN	SSCCM	GICTECFLQM	NPNST	PTQCPFC
At_DA2	CPICFLYYPSLN	SSCCM	SICTECFLQM	NPNST	PTQCPFC
Bn_DA2	CPICFLYYPSLN	SSCCM	SICTECFLQM	NPNST	PTQCPFC
At_DA2L	CPICFLYYPSLN	SSCCM	SICTECFLQM	NPNST	PTQCPFC
Gm_Glyma13g33260.1	CPICFLYYPSLN	SSCCM	SICTECFLQM	NPNST	PTQCPFC
Sb_Sb10g003820	CPICFLYYPSLN	SSCCM	SICTECFLQM	NPNST	PTQCPFC
Bd_Bradi1g49080	CPICFLYYPSLN	SSCCA	GICTECFLQM	SPTSC	PTQCPYC
Zm_gi 260935347	CPICFLYYPSLN	SSCCA	GICTECFLQM	SPTSC	PTQCPYC
Os_gi 218197613	CPICFLYYPSLN	SSCCA	GICTECFLQM	SPTSC	PTQCPYC
Vv_DA2	CPICFLYYPSLN	SSCCT	GICTECFLQM	NPNST	PTQCPYC

↑ ↑ ↑ ↑ ↑ ↑ ↑
 C C C C C H/N/T C C

Table 1: Alignment of DA2 RING domains (SEQ ID NOS:3-19).

	Pt_GI-224061326.pro	MGNKLG---RRRQVVDERYTRP	QGLYVHKDVDHKKLRKLILESKLAPCFPGDEDESCND--	55
	Rc_GI-255578534.pro	MGNKLG---RRRQVVDERYTRP	QGLYVHKDVDHKKLRKLILESKLAPCFPGDEDFGND--	55
	Vv_GI-147817790.pro	MGNKLG---RRRQVVDEKYTRP	QGLYQHKDVDHKKLRKLILDSKLAPCFPGDEEATND--	55
5	Gm_GI-356549538.pro	MGNKLG---RRRQVVDEKYTRP	QGLYNHKDVDHKKLRKLILESKLAPCFPGDEETAYD--	55
	At_GI-18411948.pro	MGNKLG---RRRQVVVEERYTKP	QGLYVNKDVDVKKLRKLILVESKLAPCFPGDESCHD--	55
	Ta_GI-408743661.pro	MGNRIGG--RRKAGVEERYTRP	QGLYEHRDIDQKKLRKLILEAKLAPCFPGADDAAGG--	56
	Hv_GI-164371454.pro	MGNRIGG--RRKAGVEERYTRP	QGLYEHRDIDQKKLRKLILETKLAPCFPGADDAAGA--	56
	Bd_GI-357140854.pro	MGNRIGG--RRKAGVEERYTRP	QGLYEHRDIDQKKLRKLILEAKLAPCFPGADDAAGG--	56
10	Os_GI-115445269.pro	MGNRIGG--RRKAGVEERYTRP	QGLYEHRDIDQKKLRKLILEAKLAPCFMGADDAAAAA--	57
	Sb_GI-242064618.pro	MGNRKGG--RPKSGGEKRFTPP	QGLYEHKDIDQKKLRKLILEAKLAPCFPGADDAAGG--	58
	Zm_GI-220961719.pro	MGNRIGG--RRKPGVEERFTRP	QGLYEHKDIDQKKLRKLILEAKLAPCFPGADDAAGGG--	58
	Ta_GI-408743658.pro	MGNRIGG--RRKAGVEERYTRP	QGLYEHRDIDQKKLRKLILEAKLAPCFPGADDAAGG--	56
	Bd_GI-357125256.pro	MGN-----	QGLYPHPDIDLKLRRLILEAKLAPCHPGSDDPRAD--	39
15	Os_GI-218197613.pro	MGNQVGGRRRRRPAVEERYTRP	QGLYPHPDIDLKLRRLILEAKLAPCFPGSDDPRAD--	58
	Zm_GI-260935347.pro	MGNQVGGRRRRRPFVDERYTRP	QGLYPHPDIDLKLRRLILEAKLAPCHPGADDARAD--	58
	Sb_GI-242092026.pro	MGNQVGGRRRRRPAVDERYTQP	QGLYPHPDIDLKLRRLILEAKLAPCHPGADDARAD--	58
		***	**** : * : * : * : * : * : * : * : *	
20	Pt_GI-224061326.pro	---HEECPICFLYYPSLNRSRCCMGKICTECFLQMKNPNSRTP	QCPFCCKTSMYAVEYRG	112
	Rc_GI-255578534.pro	---HEECPICFLYYPSLNRSRCCMGKICTECFLQMKNPNSRTP	QCPFCCKTSMYAVEYRG	112
	Vv_GI-147817790.pro	---FEECPICFLFYPSLNRSRCCMGKICTECFLQMKNPNSRTP	QCPYCKTANYAVEYRG	112
	Gm_GI-356549538.pro	---REECPICFLYYPSLNRSRCCMGKICTECFLQMKNPNSRTP	QCPFCCKTANYAVEYRG	112
	At_GI-18411948.pro	---LEECPICFLYYPSLNRSRCCMGKICTECFLQMKNPNSRTP	QCPFCCKTANYAVEYRG	112
25	Ta_GI-408743661.pro	--DLEECPICFLYYPSLNRSKCCSKGICTECFLQMKPHTARPT	QCPFCCKTANYAVEYRG	114
	Hv_GI-164371454.pro	--DLEECPICFLYYPSLNRSKCCSKGICTECFLQMKPHTARPT	QCPFCCKTANYAVEYRG	114
	Bd_GI-357140854.pro	--DLEECPICFLYYPSLNRSKCCSKGICTECFLQMKPHTARPT	QCPFCCKTANYAVEYRG	114
	Os_GI-115445269.pro	--DLEECPICFLYYPSLNRSKCCSKGICTECFLQMKPHTARPT	QCPFCCKTANYAVEYRG	115
	Sb_GI-242064618.pro	--DLEECPICFLYYPSLNRSKCCSKGICTECFLQMKPHTARPT	QCPFCCKTANYAVEYRG	116
30	Zm_GI-220961719.pro	DLDEECPICFLYYPSLNRSKCCSKGICTECFLQMKPHTARPT	QCPFCCKTANYAVEYRG	118
	Ta_GI-408743658.pro	--DLEECPICFLYYPSLNRSKCCSKGICTECFLQMKPHTARPT	QCPFCCKTANYAVEYRG	114
	Bd_GI-357125256.pro	---LDECPICFLFYPSLNRSKCCAKGICTECFLQMKSPSCTP	QCPYCKMLNYAVEYRG	96
	Os_GI-218197613.pro	---LEECPICFLFYPSLNRSKCCAKGICTECFLQMRTPSCTP	QCPYCKMASYAVEYRG	115
	Zm_GI-260935347.pro	---LDECPICFLFYPSLNRSKCCAKGICTECFLQMKSPSCTP	QCPYCKMLNYAVEYRG	115
35	Sb_GI-242092026.pro	---LDECPICFLFYPSLNRSKCCAKGICTECFLQMKSPSCTP	QCPYCKMLNYAVEYRG	115
		:*****:*****:***:*****: : : :*****:***:*****		
40	Pt_GI-224061326.pro	VKTKEEKGLEQIEEQRVIEAKIRMRQQLQDEEERMQRRLDVSSSSANIEPG-ELECGPT	171	
	Rc_GI-255578534.pro	VKTKEEKGMEQIEEQRVIEAKIRMRQQLQDEEERMQRRLDVSSSSSIAPG-EVECGSA	171	
	Vv_GI-147817790.pro	VKTKEEKGMEQIEEQRVIEAKIRMRQQLQDEEERMQRRLDVSSSSSIAPG-EVEYSTT	171	
	Gm_GI-356549538.pro	VKSKEEKGLEQIEEQRVIEAKIRMRQQLQDEEERMQRRLDVSSSSSIAPG-EVEYSTT	171	
	At_GI-18411948.pro	VKSKEEKGLEQIEEQRVIEAKIRMRQQLQDEEERMQRRLDVSSSSSIAPG-EVEYSTT	171	
	Ta_GI-408743661.pro	VKTKEERSIEQFEEQKVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	174	
	Hv_GI-164371454.pro	VKTKEERSIEQFEEQKVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	174	
45	Bd_GI-357140854.pro	VKTKEERSIEQFEEQKVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	174	
	Os_GI-115445269.pro	VKTKEERSIEQFEEQKVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	175	
	Sb_GI-242064618.pro	VKTKEERSIEQFEEQKVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	176	
	Zm_GI-220961719.pro	VKTKEERSIEQFEEQKVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	178	
	Ta_GI-408743658.pro	VKTKEERSIEQFEEQKVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	173	
50	Bd_GI-357125256.pro	VKTKEEKGVEQLEEQRVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	154	
	Os_GI-218197613.pro	VKTKEEKGTEQIEEQRVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	173	
	Zm_GI-260935347.pro	VKTKEEKGTEQIEEQRVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	173	
	Sb_GI-242092026.pro	VKTKEEKGTEQIEEQRVIEAQMRRMQQLQDEEDKMRKQSRCSSTRTIAPTTEVEYRDI	173	
		:: : ** : * : * : * : * : * : * : * : *		
55	Pt_GI-224061326.pro	TVPS-DTTPVE-----SGEIVSSQYS-----SRRPPHAGANRDDEFDLDLEDIMVMEA	218	
	Rc_GI-255578534.pro	AVQS-FRSPLE-----AEGSIPSQFS-----IRHPPHYRANRDDEFDLDLEDIMVMEA	218	
	Vv_GI-147817790.pro	AVPS-FRSPVE-----GDEIDSSQDPRAASMIITLPPRQNRDEEFDLDLEDIMVMEA	223	
	Gm_GI-356549538.pro	AVSSSVSVVE-----NDEIVSSQDSCATSVVRANATRTNRDDEFDLDLEDIMVMEA	224	

	At_GI-18411948.pro	SAIS-YNSLMD-----DGEIAPSQNAS---VVRQHSPRGNREDEVDVLEELMVMEA	220
	Ta_GI-408743661.pro	CSTS-YSVPSY--QCTEQETECCSSEFSCSAQANMRSFHSRHTRDDNIDMNIEDMMVMEA	231
	Hv_GI-164371454.pro	CSTS-YSAPPY--RCTEQETECCSSEFSCSAQANMRSFHSRHTRDGNIDMNIEDMMVMEA	231
	Bd_GI-357140854.pro	CSTS-YSVPSY--QCTEQEAECCSSEFSCSAQSNMRPFVHSRHNRRDNDIGMNIEMMVMEA	231
5	Os_GI-115445269.pro	CSTS-FSVPSY--RCAEQETECCSSEFSCSAQTSMRPFHSRHNRRDNDIDMNIEDMMVMEA	232
	Sb_GI-242064618.pro	CSTS-FSVPSY--QCTEQGNECCSSEFSCSSQANMRPFHSRHNRRDNDVDNLEEDMMVMEA	233
	Zm_GI-220961719.pro	CSTS-FSVPSY--QRTEQGNECCSSEFSCSSQANMRPFHSRHNRRDNDVDMNLEEDMMVME	235
	Ta_GI-408743658.pro	KTKEERSIEQFEEQKVIEAQMRVRQALQDEEDKMKRKQSRCS--SSMDMNIEDMMVMEA	231
10	Bd_GI-357125256.pro	GGSTPAASSA-----QGNDALLSQVQHSELLLLKNSERLKQMRNNFVDVLEEVMLMEA	208
	Os_GI-218197613.pro	GGASTTVKSSG-----QGSDDLSSQVQHAEILLKTSERLKQMRNNFMDPDEVMLVEA	227
	Zm_GI-260935347.pro	DGASTAVANSP-----RGNDVLSSEVQHSSELISRNSEAFKQMRGNNFVDVLEEVMLMEA	227
	Sb_GI-242092026.pro	DGASTGAASSP-----QGSDAISSEVQHSSELILRNSEAFKQMRGNNFVDVLEEVMLMEA	227
		.. : : : : * : : *	
15	Pt_GI-224061326.pro	IWLSIQ-ENGRQKNPLCGDAAP-----PAQYTMARYVTP---AMAPPLAGSSSSPSGG	268
	Rc_GI-255578534.pro	IWLSIQ-ENGRQKNPIYTDAAS-----SENYAVQGHYALQ---AMPP-VTESSSSPSGG	267
	Vv_GI-147817790.pro	IWLSIQ-DNGRHRNPYIGDTTT-----AEQYVTEEHYVLP---AMAP-QVESSSSPSGG	272
	Gm_GI-356549538.pro	IWLSIQ-ENGRRRNLSFVDATSGHYVADGRYVSSVSSVSS---VMGP-PTGSSSSPSGG	278
	At_GI-18411948.pro	IWLSVQ-ETGTQRNSASGEITS-----SRQYVTDNHSYVSSPPRVTPIVEPATPSSSSGG	274
20	Ta_GI-408743661.pro	IWRISIQ-EQGSIGNPACGSFMP-----FEQP-TCERQ---AFVAAPPLEIPHP-GG	276
	Hv_GI-164371454.pro	IWRISIQ-EQGSIGNPACGSFMP-----FEQP-TRERQ---AFVAASPLEIPHP-GG	276
	Bd_GI-357140854.pro	IWRISIQ-EQGSMGNPVCGNFMP-----VIEPPSRERQ---AFVPAP-LEIPHP-GG	276
	Os_GI-115445269.pro	IWRISIQ--GSIGNPVCGNFMP-----VTEPPSRERQ---PFVPAASLEIPHP-GG	276
	Sb_GI-242064618.pro	IWRISIQ-EQGHLVNPVCGSYFP-----VIEPPSRERQ---AFLPAAPLEMPHP-GG	279
25	Zm_GI-220961719.pro	IWRISIQ-EQGHLVNPVCGSYFP-----VIEPPSRERQ---AFVPAAPLEMPHP-GG	282
	Ta_GI-408743658.pro	IWRISIQ-EQGSIGNPSCGSFMP-----FEQP-TRERQ---AFVAAPLEMPHP-GG	276
	Bd_GI-357125256.pro	IWLSVQ-D--ASGNPGITGAAP-----PTIPPSYD-----TSVTASAEAAPSG-G	250
	Os_GI-218197613.pro	LWLSIQ-DQEASGNPTCGNTVS-----SVHPPRSFE-----GSMTIPAEAASSSSA	272
	Zm_GI-260935347.pro	IWLSIQ-DQEALGNPCGVSTTP-----SSIPSRPFDD-----GDMTTTAEAASSG-G	272
30	Sb_GI-242092026.pro	IWLSIQ-DQEALGNPCGVSTTP-----SSIPSRPFDD-----GAMTTTPEAASSG-G	271
		: * * : *	
	Pt_GI-224061326.pro	LACATAALAEERQQTGGES--IVHNSGNMPSFNMLPST-SSFYNRLQDADNYSPAQSSN	325
	Rc_GI-255578534.pro	LACATAALAEERQQTGGES--FAHNNENVAACNMLPGG-SSFYNRMDQDAENYSPAQSSN	324
35	Vv_GI-147817790.pro	LACATAALAEERQQMGGES--STNYNGNMPAFNMPPGS-SRFSNRVEQYPENYPIESSMD	329
	Gm_GI-356549538.pro	LACATAALAEERQQMAGESS-MSLTNENMPSENTLPGS-RRFYNRLGRDMANYPPGDNLNE	336
	At_GI-18411948.pro	LSCAISALAEERQMVGESSSHNNHNNVNVSSYSMLPGN-CDSEYDIEQEVDGIDNHHHHR-	332
	Ta_GI-408743661.pro	FSCAVAAMAEHQ-PSSMDFSYMGTSSAFFVDFMFRP-CNIAGGSMCAVE-SSPDWSWGI	333
	Hv_GI-164371454.pro	FSCAVAAMTEHQ-PSSMDFSYMGTSSAFFVDFMFRP-CNIAGGSLRAVE-SSLDWSWGI	333
40	Bd_GI-357140854.pro	FSCAVASMAEHQ-PSSMDFSYMAGSAFFVDFMFRP-CNISGGSMCAVD-SSPDWSWGI	333
	Os_GI-115445269.pro	FSCAVAAMAEHQ-PSSMDFSYMAGSAFFVDFMFRP-CNIAGGSMCNLE-SSPESWSGI	333
	Sb_GI-242064618.pro	YSCAVAALAEHQ-PASMDFSYMAGSSTYPVDFMIRP-CNMSSGSLCGVENSLLDTWSGI	337
	Zm_GI-220961719.pro	YSCAVAALAEHQ-APSMDFSYMAGSSTYPVDFMIRP-CNMSSGSPCAENSLLDTWSGI	340
	Ta_GI-408743658.pro	MDMNIEDMMVME-AIWRISIQEQ-GSIGNPSCGSFMPF-EQPTRERQAFVAAPLEMPHPG	333
45	Bd_GI-357125256.pro	FACAVAALAEQQHMLVGS--SIPATCQASKHDTLSRSDRSFTEDLSIAGSSSGTRVDES	308
	Os_GI-218197613.pro	FACAVAALAEQQQMYGEA--SSTATCHTSRCDILSRSDRSFTEDLSINGSGSSGARSEEP	330
	Zm_GI-260935347.pro	FACAVAALAEQQHMHGES--SSASPCQTIREFGTLSPDRSTTQDLVAGSSSSDSRVEEP	330
	Sb_GI-242092026.pro	FAFAVAALAEQQHMHGES--SSASACQTPRFDILSRSDRSSTEDLSVVGSSSSDSRVEEP	329
		: : :	
50	Pt_GI-224061326.pro	VLPDCRMIVTRDDGEWGADRGSDAAEAGTSYASSETAEDAGGISLLPPP--PPTDEIGG	383
	Rc_GI-255578534.pro	MLSDCRMA--RDDVQVWVADRGSDAAEAGTSYASSETEDSDGISVVLPPPLPPPPDEIVG	382
	Vv_GI-147817790.pro	ALPDGGLAVTKDDGEWGVDRGSEVAEAGTSYASSDATDEAGGVAA-----LPPTDEAEG	383
	Gm_GI-356549538.pro	EPLDEAVTMRSHGEWMDHGTQLTETATSYTNSVAAEDRGELSS-----LPRSDDNDG	390
55	At_GI-18411948.pro	-----HHYEMGETGSSNSYVSSYMTGEGFHN-----FPPPP---	363
	Ta_GI-408743661.pro	ASSCSRREVVREEGECSTDHWEAGAEAGTSYAGSDIVVDAGTTPP-----LPVTDN---	384
	Hv_GI-164371454.pro	APSGTRREVMREEGECSDHWEAGAEAGTSYAGSDIMADAGTMPP-----LPFADN---	384
	Bd_GI-357140854.pro	PPSCSR-EMIREEGECSTDHWEAGAEAGTSYAGSDIVADAGTMQ-----LPFAEN---	383
	Os_GI-115445269.pro	APSCSR-EVVREEGECSDHWEAGAEAGTSYAGSDIVADAGTMP-----LPFAEN---	383

	Sb_GI-242064618.pro	APSCSR-EVVREEGECSTDHWSEGAEGTSYAGSDIMADTGTMQP-----LPFAEN--- 387
	Zm_GI-220961719.pro	APSCSR-EVVRDEGECSADHWSEGAEGTSYAGSDIMADAGAMQP-----LPFAEN--- 390
	Ta_GI-408743658.pro	GPSCSRREVVRREEGECSTDHLSEGAEGTSYAGSDIVVDAGTMLP-----LPFADN--- 384
	Bd_GI-357125256.pro	SINRTRQTREGAEHSNN-DRWSEVADASTSCAGSDITREAGAAANL-----VASDG--- 357
5	Os_GI-218197613.pro	SSNKMHTQREGMEYSN--ERWSEMAEASSFTGSDLTTEAGAAAN-----SGG--- 375
	Zm_GI-260935347.pro	PTSNTHRTIEAAEYSNSNVQWSEVAEAGTSIAESDGTVEAGVDNS-----STSAG--- 380
	Sb_GI-242092026.pro	SSSSTHRTIEGSEYSNSNGRWSEVAEAGTSIAEADVIVEAGVGNS-----STSVG--- 379
		: : :*: :
10	Pt_GI-224061326.pro	SFQNVSGPIP-ESFEEQMMLAMAVSLAEARAMTSG--PQSAWQ 423
	Rc_GI-255578534.pro	S--DSGMIVP-ESFEEQMMLAMAVSLAEQAAMTGG--AGSAWQ 420
	Vv_GI-147817790.pro	SFQNVGGPIVPESFEEQMMLAMAVSLAEARARTS---TQGVWQ 423
	Gm_GI-356549538.pro	SLQSATEPIVPESFEEQMMLAMAVSLAEARAMSSG--QSASWQ 431
	At_GI-18411948.pro	-----PLVIVPESFEEQMMAMAVSMAEVHATTTCAPTEVTWQ 401
15	Ta_GI-408743661.pro	-YSMVASHFRPESIEEQMMYSMAVSLAEA-HGRTHT-QGLAWL 424
	Hv_GI-164371454.pro	-YSMAASHFRPESIEEQMMYSMAVSLAEA-HGRTHT-QGLTWL 424
	Bd_GI-357140854.pro	-YNMAPSHFRPESIEEQMMYSMTVSLAEA-HGRTHS-QGLAWL 423
	Os_GI-115445269.pro	-FAMAPSHFRPESIEEQMMFSMALSLADG-HGRTHS-QGLAWL 423
	Sb_GI-242064618.pro	-FTMAPSHFRPESIEEQMMFSMAVSLAEAHHGRTQA-QGLAWL 428
20	Zm_GI-220961719.pro	-FAMGPSHFRPESVVEEQMMFSMAVSLAEAHHGRTQA-QGLAWL 431
	Ta_GI-408743658.pro	-YSMVASHFRPESIEEQMMYSMAVSLAEA-HGRTHS-QGLAWL 424
	Bd_GI-357125256.pro	--SSIGSGNIPDSFEDQMMLAISLSLVDARAMASSPGPGLTWQ 398
	Os_GI-218197613.pro	--SDTGAGSIPDSFEEQMMLAMALSLADARAKASSPG--LTWR 414
	Zm_GI-260935347.pro	--SNIDSVSVDPDSFEEQMMLAMALSLVDARARAGSPG--LAWR 419
25	Sb_GI-242092026.pro	--SNIGSSSVDPDSFEEQMMLAMALSLVDARSRAGSPG--LAWR 418
		:*:*** :*:*: :

Table 2; Alignment of DA2 polypeptides (SEQ ID NOS: 20-35)

* indicates identical residues

30 : indicates conserved residues

. indicates semi-conserved residues

RING domain and first and second consensus domains are boxed.

	Si_GI-514815267.pro	-----
35	Bd_GI-357157184.pro	-----
	Br_DAlb.pro	-----
	Br_DAla.pro	-----
	At_GI-15221983.pro	-----
	Tc_GI-508722773.pro	-----
40	Gm_GI-356564241.pro	-----
	Gm_GI-356552145.pro	-----
	Vv_GI-302142429.pro	-----
	Vv_GI-359492104.pro	-----
	Sl_GI-460385048.pro	-----
45	Os_GI-218197709.pro	-----
	Os_GI-115466772.pro	-----
	Bd_GI-357160893.pro	-----
	Bd_GI-357164660.pro	-----
	Sb_GI-242092232.pro	-----
50	Zm_GI-212275448.pro	-----
	At_GI-240256211.pro	-----
	At_GI-145360806.pro	-----
	At_GI-22326876.pro	MEPPAARVTPTSIKADCSHVSNIICEETVLHSLVSHLSAALRREGISVFVDACGLQETKFF 60
	At_GI-30698242.pro	-----
55	At_GI-30698240.pro	-----
	At_GI-15240018.pro	-----
	At_GI-334188680.pro	-----

	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
	Br_DA1b.pro	-----	
5	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
	Gm_GI-356552145.pro	-----	
10	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
	Os_GI-218197709.pro	-----	
	Os_GI-115466772.pro	-----	
15	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
	At_GI-240256211.pro	-----	
20	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	SIKQNQPLTDGARVLVVVISDEVEFYDPWFPKFLKVIQGWQNNGHVVVPVIFYGVDSLTRV	120
	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	-----	
	At_GI-15240018.pro	-----	
25	At_GI-334188680.pro	-----	
	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
30	Br_DA1b.pro	-----	
	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
35	Gm_GI-356552145.pro	-----	
	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
	Os_GI-218197709.pro	-----	
40	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
45	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	YGWANSWLEAEKLTSHQSKILSNVLTDSSELVEEIVRDVYGKLYPAERVGIYARLLEIEK	180
	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	-----	
50	At_GI-15240018.pro	-----	
	At_GI-334188680.pro	-----	
	Si_GI-514815267.pro	-----	
55	Bd_GI-357157184.pro	-----	
	Br_DA1b.pro	-----	
	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	

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	Gm_GI-356564241.pro	-----
	Gm_GI-356552145.pro	-----
	Vv_GI-302142429.pro	-----
	Vv_GI-359492104.pro	-----
5	Sl_GI-460385048.pro	-----
	Os_GI-218197709.pro	-----
	Os_GI-115466772.pro	-----
	Bd_GI-357160893.pro	-----
	Bd_GI-357164660.pro	-----
10	Sb_GI-242092232.pro	-----
	Zm_GI-212275448.pro	-----
	At_GI-240256211.pro	-----
	At_GI-145360806.pro	-----
15	At_GI-22326876.pro	LLYKQHRDIRSIGIWGMPGICKTTLAKAVFNHMSSTDYDASCFIENFDEAFHKEGLHRLK 240
	At_GI-30698242.pro	-----
	At_GI-30698240.pro	-----
	At_GI-15240018.pro	-----
	At_GI-334188680.pro	-----
20		
	Si_GI-514815267.pro	-----
	Bd_GI-357157184.pro	-----
	Br_DA1b.pro	-----
	Br_DA1a.pro	-----
25	At_GI-15221983.pro	-----
	Tc_GI-508722773.pro	-----
	Gm_GI-356564241.pro	-----
	Gm_GI-356552145.pro	-----
	Vv_GI-302142429.pro	-----
30	Vv_GI-359492104.pro	-----
	Sl_GI-460385048.pro	-----
	Os_GI-218197709.pro	-----
	Os_GI-115466772.pro	-----
	Bd_GI-357160893.pro	-----
35	Bd_GI-357164660.pro	-----
	Sb_GI-242092232.pro	-----
	Zm_GI-212275448.pro	-----
	At_GI-240256211.pro	-----
	At_GI-145360806.pro	-----
40	At_GI-22326876.pro	ERIGKILKDEFDISSYIMRPTLHRDKLYDKRILVVLDVDRDSLAAESFLKRLDWFSGS 300
	At_GI-30698242.pro	-----
	At_GI-30698240.pro	-----
	At_GI-15240018.pro	-----
	At_GI-334188680.pro	-----
45		
	Si_GI-514815267.pro	-----
	Bd_GI-357157184.pro	-----
	Br_DA1b.pro	-----
50	Br_DA1a.pro	-----
	At_GI-15221983.pro	-----
	Tc_GI-508722773.pro	-----
	Gm_GI-356564241.pro	-----
	Gm_GI-356552145.pro	-----
55	Vv_GI-302142429.pro	-----
	Vv_GI-359492104.pro	-----
	Sl_GI-460385048.pro	-----
	Os_GI-218197709.pro	-----
	Os_GI-115466772.pro	-----

	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
5	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	-----	LIITTSVDKQVFQFCQINQIYTVQGLNVHEALQLFSQSVFGINEPEQNDRLSMKVIDYV 360
	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	-----	
10	At_GI-15240018.pro	-----	
	At_GI-334188680.pro	-----	
	Si_GI-514815267.pro	-----	
15	Bd_GI-357157184.pro	-----	
	Br_DA1b.pro	-----	
	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	
20	Gm_GI-356564241.pro	-----	
	Gm_GI-356552145.pro	-----	
	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
25	Os_GI-218197709.pro	-----	
	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
30	Zm_GI-212275448.pro	-----	
	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	-----	NGNPLALSIYGRELMGKKSEMETAFFELKHCPPLKIQDVLKNAYSALSDNEKNIVLDIAF 420
	At_GI-30698242.pro	-----	
35	At_GI-30698240.pro	-----	
	At_GI-15240018.pro	-----	
	At_GI-334188680.pro	-----	
40	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
	Br_DA1b.pro	-----	
	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
45	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
	Gm_GI-356552145.pro	-----	
	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
50	Sl_GI-460385048.pro	-----	
	Os_GI-218197709.pro	-----	
	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
55	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	-----	FFKGETVNVYVMQLLEESHYFPRLAIDLVDKCVLTISENTVQMNLIQDTCQEIFNGEIE 480

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	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	-----	
	At_GI-15240018.pro	-----	
5	At_GI-334188680.pro	-----	
	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
	Br_DA1b.pro	-----	
10	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
	Gm_GI-356552145.pro	-----	
15	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
	Os_GI-218197709.pro	-----	
	Os_GI-115466772.pro	-----	
20	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
	At_GI-240256211.pro	-----	
25	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	TCTRMWEPsRIRYLLEYDELEGSGETKAMPKSGLVAEHIESIFLDTSNVKFDVKHDAFKN	540
	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	-----	
	At_GI-15240018.pro	-----	
30	At_GI-334188680.pro	-----	
	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
35	Br_DA1b.pro	-----	
	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
40	Gm_GI-356552145.pro	-----	
	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
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45	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
50	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	MFNLKFLKIYNCSKYSISGLNFPKGLDSLPHYELRLLHWENYPLQSLPQDFDFGHLVKLSM	600
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	At_GI-30698240.pro	-----	
55	At_GI-15240018.pro	-----	
	At_GI-334188680.pro	-----	
	Si_GI-514815267.pro	-----	

	Bd_GI-357157184.pro	-----	
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	Br_DA1a.pro	-----	
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	Gm_GI-356552145.pro	-----	
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	Vv_GI-359492104.pro	-----	
10	Sl_GI-460385048.pro	-----	
	Os_GI-218197709.pro	-----	
	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
15	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
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	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	PYSQLHKLGTRVKDLVMLKRLILSHSLQLVECDILIYAQNIELIDLQGCTGLQRFPDTSQ	660
20	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	-----	
	At_GI-15240018.pro	-----	
	At_GI-334188680.pro	-----	
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	Bd_GI-357157184.pro	-----	
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	Br_DA1a.pro	-----	
30	At_GI-15221983.pro	-----	
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	Gm_GI-356552145.pro	-----	
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35	Vv_GI-359492104.pro	-----	
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	Os_GI-218197709.pro	-----	MGDRP 5
	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
40	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----	
45	At_GI-22326876.pro	LQNLRVVNLSGCTEIKCFSGVPPNIEELHLQGTIRIREIPIFNATHPPKVKLDRKKLWNLL	720
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	At_GI-30698240.pro	-----	
	At_GI-15240018.pro	-----	
	At_GI-334188680.pro	-----	
50			
	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
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55	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
	Gm_GI-356552145.pro	-----	

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	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
	Os_GI-218197709.pro	DMGAGVALRFSHNDWTLEEDSKALHFLQPDVLVLTGDYGNENVQLVKSISDLQLPKAAIL	65
5	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
10	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	ENFSDVEHIDLECVTNLATVTSNNHVMGKLVCLNMKYCSNLRGLPDMVSLESILKVLYLSG	780
	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	-----	
15	At_GI-15240018.pro	-----	
	At_GI-334188680.pro	-----	
	Si_GI-514815267.pro	-----	
20	Bd_GI-357157184.pro	-----	
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	Br_DA1a.pro	-----	
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	Tc_GI-508722773.pro	-----	
25	Gm_GI-356564241.pro	-----	
	Gm_GI-356552145.pro	-----	
	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
30	Os_GI-218197709.pro	GNHDCWHTYQFSEKKVDRVQLQLESIGEQHVGKCLDFPTIKLSVVGGRPFSCGGNRIFR	125
	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
35	Zm_GI-212275448.pro	-----	
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	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	CSELEKIMGFPRNLKKLYVGGTAIRELPQLPNSLEFLNAHGCKHLKSINLDFEQLPRHFI	840
	At_GI-30698242.pro	-----	
40	At_GI-30698240.pro	-----	
	At_GI-15240018.pro	-----	
	At_GI-334188680.pro	-----	
45	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
	Br_DA1b.pro	-----	
	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
50	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
	Gm_GI-356552145.pro	-----	
	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
55	Sl_GI-460385048.pro	PKLLSKWYGVNDMAESAKRIYDAATNAPKEHAVILLAHNGPTGLGSRMEDICGRDWVAGG	185
	Os_GI-218197709.pro	-----	
	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	

	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----	
5	At_GI-22326876.pro	FSNCYRFSSQVIAEFVEKGLVASLARAKQEELIKAPEVIICIPMDTRQRSSFRLQAGRNA	900
	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	-----MPISDVASLVGGAALGAPLSE	21
	At_GI-15240018.pro	-----MASDIYSSDDEGFGEKVGLIG	21
	At_GI-334188680.pro	-----MWCLSCFKPSTKHDP	15
10			
	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
	Br_DA1b.pro	-----	
15	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
	Gm_GI-356552145.pro	-----	
20	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
	Os_GI-218197709.pro	GDHGDPLDLEQAISDLQRETGVSIPLVVFHGMHKSLAYGRGLRKMIAFGANRTIYLNGAVV	245
	Os_GI-115466772.pro	-----	
25	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
	At_GI-240256211.pro	-----	
30	At_GI-145360806.pro	-----	
	At_GI-22326876.pro	MTDLVPWMQKPISGFSMSVVVSFQDDYHNDVGLRIRC VG TWKTWNNQPDRIVERFFQCWA	960
	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	IFKLVIEEAKKVKDFKP-----L	39
	At_GI-15240018.pro	EKDRFEAETIHVIEVSQ-----H	39
35	At_GI-334188680.pro	SEDRFEETNIVTGIS-----	31
	Si_GI-514815267.pro	-----	
	Bd_GI-357157184.pro	-----	
40	Br_DA1b.pro	-----	
	Br_DA1a.pro	-----	
	At_GI-15221983.pro	-----	
	Tc_GI-508722773.pro	-----	
	Gm_GI-356564241.pro	-----	
45	Gm_GI-356552145.pro	-----	
	Vv_GI-302142429.pro	-----	
	Vv_GI-359492104.pro	-----	
	Sl_GI-460385048.pro	-----	
	Os_GI-218197709.pro	PRVNHAQSSRQPAISTSEKTGLEGLTGLMVPTSRAFTIVDLFEGAVEKISEVWVTVGDAR	305
50	Os_GI-115466772.pro	-----	
	Bd_GI-357160893.pro	-----	
	Bd_GI-357164660.pro	-----	
	Sb_GI-242092232.pro	-----	
	Zm_GI-212275448.pro	-----	
55	At_GI-240256211.pro	-----	
	At_GI-145360806.pro	-----MDSSSSSSSSSPSSSYGVARVS	22
	At_GI-22326876.pro	PTEAPKVVDHIFVLYDTKMHPSDSEENHISMWAHEVKFEFHTVSGENNP LGASCKVTEC	1020
	At_GI-30698242.pro	-----	
	At_GI-30698240.pro	SQDLASTMERLVPIFNEIDMMQOGSNRGTSSELKVL TETMERAGEMVHKCSRIQWYSIAKK	99

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At_GI-15240018.pro EADIQKAKQORSLATHEAEKLDLATHEAEQLDLAIQEFSSRQEEEEERRRTRELENDQAQIAN 99
 At_GI-334188680.pro -----LYEDVILRQRREADQIEWAIQDSFNPQE-----TSRCRQREEDDQIAR 75

5 Si_GI-514815267.pro -----MGWLSKIFKGSVN-RVSRGHYNGNSHE-----GYS 29
 Bd_GI-357157184.pro -----MGWLNKIFKGSVN-RVSRGNYDGNWHD-----GNS 29
 Br_DA1b.pro -----MGWLNKIFKGSNQ-RHPLGNEHYHHNGGYYENYP 33
 Br_DA1a.pro -----MGWLNKIFKGSNQ-RHPLGNDHDHN-----GYYSYP 31
 At_GI-15221983.pro -----MGWLNKIFKGSNQ-RLRVGNKHNHN-VYYDNYP 32

10 Tc_GI-508722773.pro -----MDWIKKIFKGAH-KFSEG-----HHHG-----NYV 25
 Gm_GI-356564241.pro -----MGWLSRIFKGSNDHNLSEGHYYKEDA-----GYY 29
 Gm_GI-356552145.pro -----MGWLSRIFKGSNDHNLSEGHYYKEDA-----GYY 29
 Vv_GI-302142429.pro -----MGWLNKIFKGSNH-KISEGNYHGRIYQ-----GDT 28
 Vv_GI-359492104.pro -----MGWLNKIFKGSNH-KISEGNYHGRIYQ-----GDT 28

15 Sl_GI-460385048.pro -----MGWLNKIFRGSNH-KISEGQYDWRCE-----GHT 28
 Os_GI-218197709.pro TELEQELVLYKQPHKSVPSNIAIWSTMGWLTKEFRGSGTH-KISEGQYHSPAEETIWNTP 364
 Os_GI-115466772.pro -----MGWLTKEFRGSGTH-KISEGQYHSPAEETIWNTP 33
 Bd_GI-357160893.pro -----MGWLTKEFRGSGTH-KISEGQYHSPAEETIWNTP 33
 Bd_GI-357164660.pro -----MGWLTKEFRGSGTH-KISEGQYHSPAEETIWNTP 33

20 Sb_GI-242092232.pro -----MGWLTKEFRGSGTH-KISEGQYHSPAEETIWNTP 33
 Zm_GI-212275448.pro -----MGWLTKEFRGSGTH-KISEGQYHSPAEETIWNTP 33
 At_GI-240256211.pro -----MGWLTKEFRGSGTH-KISEGQYHSPAEETIWNTP 33
 At_GI-145360806.pro HISNPCIFGEVGSSSSSTYRDKKWKLMKWSKLFKSGSNGGSGAHTNHHPPQFQEDENM 82
 At_GI-22326876.pro GVEVITAATGDTSVSGIIRESETITIEKEDTIDEEDTPLSRKPEETNRSRSSLQ 1080

25 At_GI-30698242.pro -----ALYTREIKA--INQDFLKFCQIELQLIQRNLQYMRSMGMASVSTKADLLSDIGNEFSK 157
 At_GI-15240018.pro VLQHEERE-----RLINKKTALEDEDELLARTLEESLKNNRRKMFEEQVNDQ 150
 At_GI-334188680.pro GLQYVEET-----ELDKSVDEED-----QQ 96

30 Si_GI-514815267.pro TQHTKSY----- 36
 Bd_GI-357157184.pro SENIR----- 34
 Br_DA1b.pro -HEHS-----EPSAETDA-----DHT 48
 Br_DA1a.pro -HDEPSADTDPPDPDPDE-----THT 52

35 At_GI-15221983.pro TASHDEPSAADTDADNDP-----HHT 55
 Tc_GI-508722773.pro EDPHP-----QF 32
 Gm_GI-356564241.pro LPSTS----- 34
 Gm_GI-356552145.pro LPSTS----- 34
 Vv_GI-302142429.pro VQNEP----- 33
 Vv_GI-359492104.pro VQNEP----- 33

40 Sl_GI-460385048.pro EEDDP----- 33
 Os_GI-218197709.pro SNSAVVTMVPLESTFGQLDLLLLATDLRQLVIDDVCCKLRQQAQVPLHLMYSQQLQLQ 424
 Os_GI-115466772.pro SNSAVVT----- 40
 Bd_GI-357160893.pro SSSTVVT----- 40
 Bd_GI-357164660.pro SSSTAVN----- 40

45 Sb_GI-242092232.pro SSSPVVT----- 40
 Zm_GI-212275448.pro SSSPVVT----- 40
 At_GI-240256211.pro RYSAEGSDFDKKEIECAIALSLS-----EQEHVIPQDDKGKKIIE 73
 At_GI-145360806.pro VFPLPPS----- 89

50 At_GI-22326876.pro LSSTSSKVRSKGNVFWKWLGCFF-----LQPKNLRSRRTTALLEA 1122
 At_GI-30698242.pro -----LCLVAQPEVVTKEFWLKRPLMELKKMLFEDGV-----VTVVVSAPYALGKTTLVTK 207
 At_GI-30698240.pro LALIVQESLNMEEYPIR-LEEYK-----SISRRAPLDVDEQ-FAKA 189
 At_GI-15240018.pro LSKIVEESLKE----- 107

55 Si_GI-514815267.pro -----GAHGNEDE-----DMDHAIALSLSEQDQRKGKAIDTEHHLDE-----ED 74
 Bd_GI-357157184.pro -----GAYDESDNE-----DIDRAIALSLAEEDENKGAIDPDYS----- 70
 Br_DA1b.pro QEPSTSEETWNGKENE-----EVDRIALSILEE-ENQRPETNTG----- 88

	Br_DA1a.pro	QEPSTSEEDTS-GQENE-----DIDRAIALSLIENSQGGTNNNTCAAN-----	93
	At_GI-15221983.pro	QEPSTSEDNTSNDQENE-----DIDRAIALSLLEE--NQEQTSISG-----	94
	Tc_GI-508722773.pro	NAPSVS-GDAWQELENE-----DVDRAIALSLLGE--SQKGRKVID-----	70
	Gm_GI-356564241.pro	GVTN-----NQNE-----DIDRAIALSLVEESRRANNNVNGER-----	69
5	Gm_GI-356552145.pro	GVTNDAWNQSQNQNE-----DIDRAIALSLVEETQKANNNVN-----	73
	Vv_GI-302142429.pro	----SCSGDVWAETENE-----DIDRAIALSLSEE--EQKGGKVID-----	68
	Vv_GI-359492104.pro	----SCSGDVWAETENE-----DIDRAIALSLSEE--EQKGGKVIDE-----	69
	Sl_GI-460385048.pro	----STAEDSWSEIE-----EIDRAIALSLSEE--EQKGIKVID-----	66
	Os_GI-218197709.pro	TSHAHQHGDVPSEFDNE-----DIARAISSLLEEEQRKAKAIEKD-----	465
10	Os_GI-115466772.pro	-----DVPSEFDNE-----DIARAISSLLEEEQRKAKAIEKD-----	73
	Bd_GI-357160893.pro	-----DVLSEFDNE-----DIDRAIALSLSEE--QRKSKGTGKD-----	72
	Bd_GI-357164660.pro	-----YALSEFDNE-----DIDRAIALSLSEEQRKSKGTGKD-----	73
	Sb_GI-242092232.pro	-----DIFSEFNNE-----DIDRAIALSLSEEEQRKAKTIDKD-----	73
	Zm_GI-212275448.pro	-----DILSEFNNE-----DIDRAIALSLSEEEQRKEKAIDKD-----	73
15	At_GI-240256211.pro	YKSETEEDDDDDDEDEDEEYMQLEAAEEEEERRVAQAQIEEEEKRRAAEQLEETEKLLAK	133
	At_GI-145360806.pro	----SLDDRSRGARDKE-----ELDRSISLSLADN-TKRPHGYGWS-----	125
	At_GI-22326876.pro	LEEALKEREKLEDTREI-----QIALIESKKIKKIQADERDQIKHADER----	1167
	At_GI-30698242.pro	----MVRKRQEEDKEI-----EIERVKESLKLAKQAEKRRLEESKEQ----	41
	At_GI-30698240.pro	LCHDADVKEKFKQIFFI-----SVSKFPNVRLIGHKLEHIGCKANEYEN----	252
20	At_GI-15240018.pro	VKESLKNKGKQKQFEDE-----QVKKDEQLALIVQESLNMVESPRLLEN----	234
	At_GI-334188680.pro	-----KGKSKQFEDD-----QVENDEQQALMVQESLYMVVLSAQLEED----	145
	Si_GI-514815267.pro	EQLARALQENTSPRTLDEDEQLAR-----ALQESMNDEHP	108
25	Bd_GI-357157184.pro	-----LEEDEQLAR-----ALHESLNTGSP	90
	Br_DA1b.pro	-----AWKHAM-MDDDEQLAR-----AIQESMIARN-	113
	Br_DA1a.pro	-----AGKYAM-VDEDEQLAR-----AIQESMVVGNT	119
	At_GI-15221983.pro	-----KYSMPVDEDEQLAR-----ALQESMVVGNS	119
	Tc_GI-508722773.pro	-----DEYQLEEDQLAR-----ALQESLNFEPP	94
30	Gm_GI-356564241.pro	-----ILSLQTLLEEDQLAR-----AIEQSLNLESP	96
	Gm_GI-356552145.pro	-----DYRSQLEEDQLAR-----AIEQSLNLESP	98
	Vv_GI-302142429.pro	-----NEFQLEEDQLAR-----AIQESLNLESP	92
	Vv_GI-359492104.pro	-----L-DNEFQLEEDQLAR-----AIQESLNLESP	95
	Sl_GI-460385048.pro	-----SESQLEEDQLAR-----ALQESLNLESP	90
35	Os_GI-218197709.pro	-----MHLEEDQLAR-----AIQESLNLESP	487
	Os_GI-115466772.pro	-----MHLEEDQLAR-----AIQESLNLESP	95
	Bd_GI-357160893.pro	-----LHLEDEDEQLAR-----AIHESLNLESP	94
	Bd_GI-357164660.pro	-----QHLEDEDEQLAR-----AIQESLNLESP	95
	Sb_GI-242092232.pro	-----MHLEEDQLAR-----AIQESLNLESP	95
40	Zm_GI-212275448.pro	-----MHLEEDQLAR-----AIQESLNLESP	95
	At_GI-240256211.pro	ARLEEEEMRRSKAQLEEDELLAK-----ALQESMNVGSP	167
	At_GI-145360806.pro	-----MDNNRDFFR-----PFHGGLNPSSF	145
	At_GI-22326876.pro	-----EQRKHSKDHEEEIEESNEKEERRHSDYVIEELVLKGGKGRQLDDDKADEKEQ	1221
	At_GI-30698242.pro	-----GKRIQVDDD-----QLAKTTSKDKGQ	62
45	At_GI-30698240.pro	-----DLDAMLYIQQLKQLGRNGSILLVLDV-----WAEESLLQKFL	292
	At_GI-15240018.pro	-----NNISTRAPVDEDEQLAK-----AVEESLKGKGQ	262
	At_GI-334188680.pro	-----KNISTIPPLNEDAQLQK-----VIWESAKGKGQ	173
50	Si_GI-514815267.pro	PR-----QHIPIEDVHSESAPASSLPYPVFTNGSRVCA	142
	Bd_GI-357157184.pro	PH-----QNVVVDVPSERVEPTREPPPPVFLSSGFRACA	124
	Br_DA1b.pro	----GTT-----YDFGNAY-----GNGHMHGGGNVYDNGDIYYPRPIAFSMDFRICA	156
	Br_DA1a.pro	PRQKHGSS-----YDIGNAYGAGDVYGNHMHGGGNVYANGDIYYPRPTAFFMDFRICA	173
	At_GI-15221983.pro	PRHKS GST-----YDNGNAYGAGDLYGNHMYGGGNVYANGDIYYPRPITFQMDFRICA	173
55	Tc_GI-508722773.pro	P-----QYENANMYQMPVHFPMGYRICA	118
	Gm_GI-356564241.pro	P-----RYGNENMYQPPIQYFPLG--ICA	118
	Gm_GI-356552145.pro	P-----RYGNENMYQPPIQYFPMGSRICA	122
	Vv_GI-302142429.pro	PQ-----HGNGN-----GNGNIYQPIPFYSTGFRICA	120
	Vv_GI-359492104.pro	PQ-----HGNGN-----GNGNIYQPIPFYSTGFRICA	123

	Sl_GI-460385048.pro	PQ-----HVSRRNDHGGGNVYGNNGNFYHPVFPFYSASFVCA	126	
	Os_GI-218197709.pro	-----PRARENGNANGGNMYQPLPFMFSSGFRTCA	517	
	Os_GI-115466772.pro	-----PRARENGNANGGNMYQPLPFMFSSGFRTCA	125	
	Bd_GI-357160893.pro	PCARDNGSPPH---ARDNSSPPHARENSSHPARENGIANGGNSIQHSPFMFSSGFRTCA	151	
5	Bd_GI-357164660.pro	-----PRAREKSSHPARENGSANGGNSYQL-PLMFSSGFRTCA	133	
	Sb_GI-242092232.pro	P-----PSRENGSANGGNAYHPLPFMFSSGFRACA	125	
	Zm_GI-212275448.pro	PRRNGSAN-----GGTMYHPPRETGNAYQPPRENGSANGGNAYHPLPFMFSSGFRACA	148	
	At_GI-240256211.pro	P-----RYDPGNILQPYPFLIPSSSHRICV	191	
	At_GI-145360806.pro	IP-----PYEPSYQYRRRQRICG	163	
10	At_GI-22326876.pro	IKH-----SKDHVEE-----EVNPPLSKCK	1241	
	At_GI-30698242.pro	INH-----SKDVVEE-----DVNPPPS--I	80	
	At_GI-30698240.pro	IQLPDYKILVTSRFEFTSFGPTFHLKPLIDDEVECRDEIEENEKLP----	EVNPPLSMCG	348
	At_GI-15240018.pro	IKQ-----SKDEVEGDGMLL-----ELNPPPSLCG	287	
	At_GI-334188680.pro	IEH-----FKDPVEEDGNLPRVDLNVNHPHSICD	202	
15				
	Si_GI-514815267.pro	GCKTPIGQGRFLSCMDSVWHPQCFRCYGCIDIPISEYEFVHE---DHAYHRSCYKERF-H	198	
	Bd_GI-357157184.pro	GCNNPIGNRFLSCMDSVWHPQCFRCFACNKPISEYEFAMHE---NQPYHKSCYKDF-H	180	
	Br_DAlb.pro	GCNMEIGHGRYLNCLNALWHPQCFRCYGCSPHISEYEFSTSG---NYPFHKACYRERF-H	212	
20	Br_DAla.pro	GCNMEIGHGRYLNCLNALWHPQCFRCYGCSPHISEYEFSTSG---NYPFHKACYRERY-H	229	
	At_GI-15221983.pro	GCNMEIGHGRFLNCLNSLWHPQCFRCYGCSPHISEYEFSTSG---NYPFHKACYRERY-H	229	
	Tc_GI-508722773.pro	GCNTEIGHGRFLNCLNAFWHPQCFRCHACNLPISDYEFMSG---NYRPHKSCYKERY-H	174	
	Gm_GI-356564241.pro	GCYTEIGFGRYLNCLNAFWHPQCFRCHACNLPISDYEFSTSG---NYPYHKSCYKESY-H	174	
	Gm_GI-356552145.pro	GCYTEIGYGRYLNCLNAFWHPQCFRCHACNLPISDYEFSTSG---NYPYHKSCYKESY-H	178	
25	Vv_GI-302142429.pro	GCNTEIGHGRFLSCMGAVWHPQCFRCHGCGYPISDYEYSMNG---NYPYHKSCYKEHY-H	176	
	Vv_GI-359492104.pro	GCNTEIGHGRFLSCMGAVWHPQCFRCHGCGYPISDYEYSMNG---NYPYHKSCYKEHY-H	179	
	Sl_GI-460385048.pro	GCSTEIGHGRFLSCMGAVWHPQCFRCHACNQPISDYEFMSG---NYPYHKTCYKEHY-H	182	
	Os_GI-218197709.pro	GCHSEIGHGRFLSCMGAVWHPQCFRCHACNQPISDYEFMSG---NHPYHKTCYKERF-H	573	
	Os_GI-115466772.pro	GCHSEIGHGRFLSCMGAVWHPQCFRCHACNQPISDYEFMSG---NHPYHKTCYKERF-H	181	
30	Bd_GI-357160893.pro	GCHSEIGHGRFLSCMGAVWHPQCFCHACSQPIYDYEFMSG---NHPYHKTCYKERF-H	207	
	Bd_GI-357164660.pro	GCHSEIGHGRFLSCMGAVWHPQCFCHGCSQPIYDYEFMSG---NHPYHKTCYKERF-H	189	
	Sb_GI-242092232.pro	GCHREIGHGRFLSCMGAVWHPQCFRCHACSQPIYDYEFMSG---NHPYHKTCYKEQF-H	181	
	Zm_GI-212275448.pro	GCHREIGHGRFLSCMGAVWHPQCFRCHACSQPIYDYEFMSG---NHPYHKTCYKEQF-H	204	
	At_GI-240256211.pro	GQQAIEIGHGRFLSCMGVWHPQCFCCNACDKPIIDYEFMSG---NRPYHKLCYKEQH-H	247	
35	At_GI-145360806.pro	GCNSDIGSGNYLGCMTFFHPECFRCHSCGYAITEHEFSLSG---TKPYHKLCKFKELT-H	219	
	At_GI-22326876.pro	DCKSAIEDGISINAYGSVWHPQCFCLRCREPIAMNEISDLR---GMYHKPCYKELR-H	1296	
	At_GI-30698242.pro	DGKSEIGDGTSVN-----PRCLCCFCHCRPFVMEILKK-----GKFHIDCYKEYRN	128	
	At_GI-30698240.pro	GCNSAVKHEESVNILGVLWHPGCFCCRSCDKPIAHELENHVSNSRGKFHKSCYER----	404	
	At_GI-15240018.pro	GCNFAVEHGGSVNILGVLWHPGCFCCRACHKPIAHDENHVSNSRGKFHKSCYER----	343	
40	At_GI-334188680.pro	GCKSAIEYGRSVHALGVNWHPECFCCRYCDKPIAMHEFS---NTKGRCHITCYERSH--	256	
		* * * * *		
	Si_GI-514815267.pro	PKCDVCNSFIPTNKNGLIEYRAHPFWQKYCPSHENDGTPRCCSCERMEPKHSQYITLDD	258	
	Bd_GI-357157184.pro	PKCDVCKDFIPTNKDGLIEYRAHPFWQKYCPSHEDDGTPrCCSCERMEDTDIKYIRLDD	240	
45	Br_DAlb.pro	PKCDVCSLFISTNHAGLIEYRAHPFWVQKYCPSHEHDATPRCCSCERMENRNTGYFELND	272	
	Br_DAla.pro	PKCDVCSLFIPTNHAGLIEYRAHPFWVQKYCPSHEHDATPRCCSCERMENRNTGYVELND	289	
	At_GI-15221983.pro	PKCDVCSHFIPTNHAGLIEYRAHPFWVQKYCPSHEHDATPRCCSCERMENRNTRYVELND	289	
	Tc_GI-508722773.pro	PKCDVCNDFIPTNPAGLIEYRAHPFWIQKYCPSHEHDSTPRCCSCERMENPQDTGYVALND	234	
	Gm_GI-356564241.pro	PKCDVCKHFIPTNPAGLIEYRAHPFWIQKYCPTHEHDGTPrCCSCERMESQEAGYIALKD	234	
50	Gm_GI-356552145.pro	PKCDVCKHFIPTNPAGLIEYRAHPFWIQKYCPTHEHDGTTPrCCSCERMESQEAGYIALKD	238	
	Vv_GI-302142429.pro	PKCDVCKHFIPTNPAGLIEYRAHPFWVQKYCPSHEHDTPrCCSCERMENPRDTRYVALND	236	
	Vv_GI-359492104.pro	PKCDVCKHFIPTNPAGLIEYRAHPFWVQKYCPSHEHDTPrCCSCERMENPRDTRYVALND	239	
	Sl_GI-460385048.pro	PKCDVCKHFIPTNAAGLIEYRAHPFWSQKYCPTHEHDGTPrCCSCERMENPRDTRYIALDD	242	
	Os_GI-218197709.pro	PKCDVCKQFIPITNMNGLIEYRAHPFWLQKYCPSHEVDGTPrCCSCERMENPRESRYVLLDD	633	
55	Os_GI-115466772.pro	PKCDVCKQFIPITNMNGLIEYRAHPFWLQKYCPSHEVDGTPrCCSCERMENPRESRYVLLDD	241	
	Bd_GI-357160893.pro	PKCDVCKQFIPITNMNGLIEYRAHPFWLQKYCPSHEVDGTPrCCSCERMENPRESRYVLLDD	267	
	Bd_GI-357164660.pro	PKCDVCKQFIPITNTNGLIEYRAHPFWLQKYCPSHEVDGTPrCCSCERMENPRESRYVLLDD	249	
	Sb_GI-242092232.pro	PKCDVCKQFIPITNMNGLIEYRAHPFWLQKYCPSHEVDGTPrCCSCERMENPRESRYVLLDD	241	
	Zm_GI-212275448.pro	PKCDVCKQFIPITNMNGLIEYRAHPFWQKYCPSHEMDGTPrCCSCERMENPRESKYVLLDD	264	

	At_GI-240256211.pro	PKCDVCHNFIFITNPAGLIEYRAHPFWMQKYCPSHERDGTPRCCSCERMEDTKYLILDD	307
	At_GI-145360806.pro	PKCEVCHHFIPITNDAGLIEYRCHPFWNQKYCPSHEYDKTARCCSCERLESWDVRYITLED	279
	At_GI-22326876.pro	PNCYVCEKKIPRTAEG-L-KYHEHPFWMETCYCPSHDGDGTPKCCSCERLEHCGTQYVMLAD	1355
	At_GI-30698242.pro	RNCYVQQKIPVNAEGIRKFSEHPFWEKEYCPIHDEDTAKCCSCERLEPRGTNYVMLGD	188
5	At_GI-30698240.pro	-YCYVCKEKK-----MKTYNHPFWEERYCPVHEADGTPKCCSCERLEPRGTNYVMLGD	457
	At_GI-15240018.pro	-YCYVCKEKK-----MKTYNHPFWEERYCPVHEADGTPKCCSCERLEPRGTNYVMLAD	396
	At_GI-334188680.pro	PNCHVCKKKFP-----GRKYKEHPFWEKEYCFFHEVDGTPKCCSCERLEPWGTYVMLAD	311
		* * * : * * * : * * : * * * * * * * *	
10	Si_GI-514815267.pro	GRRLCLECLHTAIDMTNECQPLYIDIQEFYEGMNMKVEQQVPLLLVERQALNEAMEAEKI	318
	Bd_GI-357157184.pro	GRKLCLECLTSATMDSPECQHLMDIQEFFEGLNMKVEQQVPLLLVERQALNEALEAEKS	300
	Br_DA1b.pro	GRKLCLECLDSSVMDTFQCQPLYLQIQEFYEGMNMKVEQQVPLLLVERQALNEAREGERN	332
	Br_DA1a.pro	GRKLCLECLDSAVMDTFQCQPLYLQIQEFYEGMNMKVEQQVPLLLVERQALNEAREGEKN	349
	At_GI-15221983.pro	GRKLCLECLDSAVMDTMQCQPLYLQIQEFYEGMNMKVEQQVPLLLVERQALNEAREGEKN	349
15	Tc_GI-508722773.pro	GRKLCLECLDSAVMDTKQCQPLYLDILEFYEGMNMKVEQQVPLLLVERQALNEAREGEKN	294
	Gm_GI-356564241.pro	GRKLCLECLDSSIMDTNECQPLHADIQRFYDSLNMKLDQQIPLLLVERQALNEAREGEKN	294
	Gm_GI-356552145.pro	GRKLCLECLDSAIMDTNECQPLHADIQRFYDSLNMKLDQQIPLLLVERQALNEAREGEKN	298
	Vv_GI-302142429.pro	GRKLCLECLDSAIMDTNECQPLYLDIQEFYEGMNMKVEQQVPLLLVERQALNEAMEGEKS	296
	Vv_GI-359492104.pro	GRKLCLECLDSAIMDTNECQPLYLDIQEFYEGMNMKVEQQVPLLLVERQALNEAMEGEKS	299
20	Sl_GI-460385048.pro	GRKLCLECLDSAIMDTSQCQPLYLDIQEFYEGMNMKVEQQVPLLLVERQALNEAMDERH	302
	Os_GI-218197709.pro	GRKLCLECLDSAVMDTSECQPLYLEIQEFYEGMNMKVEQQVPLLLVERQALNEAMEGEKT	693
	Os_GI-115466772.pro	GRKLCLECLDSAVMDTSECQPLYLEIQEFYEGMNMKVEQQVPLLLVERQALNEAMEGEKT	301
	Bd_GI-357160893.pro	GRKLCLECLDSAVMDTTECQPLYLEIQEFYEGMNMKVEQQVPLLLVERQALNEAMEGEKT	327
	Bd_GI-357164660.pro	GRKLCLECLDSAVMDTTECQPLYLEIQEFYEGMNMKVEQQVPLLLVERQALNEAMEGEKT	309
25	Sb_GI-242092232.pro	GRKLCLECLDSAVMDTNECQPLYLEIQEFYEGMNMKVEQQVPLLLVERQALNEAMEGEKA	301
	Zm_GI-212275448.pro	GRKLCLECLDSAVMDTNDQCQPLYLEIQEFYEGMNMKVEQQVPLLLVERQALNEAMEGEKA	324
	At_GI-240256211.pro	GRKLCLECLDSAIMDTHECQPLYLEIREFYEGMNMKVEQQIPMLLVERSALNEAMEGEKH	367
	At_GI-145360806.pro	GRSLCLECMETAITDTGECQPLYHAIRDYEGMYMKLDQQIPMLLVQREALNDAIVGEKN	339
	At_GI-22326876.pro	FRWLCRECMDSAIMDSDECQPLHFEIREFFEGMNMKVEQQVPLLLVERQALNEAMEGEKI	1415
30	At_GI-30698242.pro	FRWLCEICMGSAMVMDTNEVQPLHFEIREFFEGMNMKVEQQVPLLLVERQALNEAMEGEKI	248
	At_GI-30698240.pro	GRWLCEICG-KSAMDSDECQPLYFDMRDFEGLNMKIEKEFPFLILVRKELNKK--KEEKI	514
	At_GI-15240018.pro	GRWLCEICMNSAMVMDSECCQPLHFEIREFFEGMNMKIEKEFPFLILVRKELNKK--KEEKI	456
	At_GI-334188680.pro	NRWLCEICMNSAMVMDIYECQPLHFEIREFFEGMNMKIEKEFPFLILVRKELNKK--KEEKI	371
		* * * : * * : * * : : : : : : : : : * * : * * : *	
35		↓	
	Si_GI-514815267.pro	G-HHLP---ETRGLCLSEEQIVRTILRRPII-GPGNRIIDMITGPKLVRRCEVTAILIL	373
	Bd_GI-357157184.pro	G-HHLP---ETRGLCLSEEQIVRTILRRPTI-GPGNRIIDMITGPKLVRRCEVTAILIL	355
	Br_DA1b.pro	GHHMP---ETRGLCLSEEQIVRTILRRPSK---GNWSGNMITEQFKLTRCEVTAILIL	385
40	Br_DA1a.pro	GHHMP---ETRGLCLSEEQIVRTILRRPSK-GTGNWAGNMITEPYKLTRCEVTAILIL	405
	At_GI-15221983.pro	GHHMP---ETRGLCLSEEQIVRTILRRPSK-GTGNWAGN-ITEPYKLTRCEVTAILIL	404
	Tc_GI-508722773.pro	GHHMP---ETRGLCLSEEQIVRTILRRPRF-GTGNRAMDMITEPCKLTRCEVTAILIL	350
	Gm_GI-356564241.pro	GHHMP---ETRGLCLSEE--LSTFSRRPRL-G---TAMDMRAQPYRPTTRCDVTAILVL	345
	Gm_GI-356552145.pro	GHHMP---ETRGLCLSEE--LSTFSRRPRL-G---TTMDMRAQPYRPTTRCDVTAILIL	349
45	Vv_GI-302142429.pro	GHHMP---ETRGLCLSEEQIVRTILRRPKI-GTGNRVNMNITEPCKLTRCDVTAILIL	352
	Vv_GI-359492104.pro	GHHMP---ETRGLCLSEEQIVRTILRRPKI-GTGNRVNMNITEPCKLTRCDVTAILIL	355
	Sl_GI-460385048.pro	GHHMP---ETRGLCLSEEQIVRTILRRPRI-GAGNRVMDMRITEPYKLTRCEVTAILIL	358
	Os_GI-218197709.pro	GHHLP---ETRGLCLSEEQIVRTILRRPRM-AGN-KVMEMITEPYRLTRCEVTAILIL	748
	Os_GI-115466772.pro	GHHLP---ETRGLCLSEEQIVRTILRRPRM-AGN-KVMEMITEPYRLTRCEVTAILIL	356
50	Bd_GI-357160893.pro	GHHLP---ETRGLCLSEEQIVRTILRRPRM-TGN-KIMEMITEPYRLTRCEVTAILIL	382
	Bd_GI-357164660.pro	GHHLP---ETRGLCLSEEQIVRTILRRPRM-AGN-KIMEMRITEPYRLTRCEVTAILIL	364
	Sb_GI-242092232.pro	GHHLP---ETRGLCLSEEQIVRTILRRPRM-AGN-KIMEMRITEPYRLTRCEVTAILIL	356
	Zm_GI-212275448.pro	GHHLP---ETRGLCLSEEQIVRTILRRPRI-PRM-AGN-KIMEMRITEPYRLTRCEVTAILIL	378
	At_GI-240256211.pro	GHHLP---ETRGLCLSEEQIVRTILRRPRI-GAGYKLIDMITEPCLIRCEVTAILIL	423
55	At_GI-145360806.pro	GHHMP---ETRGLCLSEEQIVTSVLRPRPRL-G-AHRLVGMRTQPRQLTRKCEVTAILVL	394
	At_GI-22326876.pro	DKQGDQCLMVVRGICLSEEQIVTSVSGVRR-MLNKQILDVTESQVRVVRKCEVTAILIL	1474
	At_GI-30698242.pro	DYHR---AAVTRGLCMSEEQIVPSIIKGPMPDNQLITDIVTESQVRV-GFEVTGILII	304
	At_GI-30698240.pro	DNHY---EVLIRAYCMSEQIMTYVSEEPRT-GQNKQLIDMDTEPQGVVHECKVTAILIL	570
	At_GI-15240018.pro	DYQY---EVVTRGICLSEEQIVDSVSGVRR-CPNNKLVGMATESQVTRCEVTAILIL	512

At_GI-334188680.pro DNHQ--GVVTRGICLSEGGQIVNSVFKKPTM-GPNGELVSLGTEPQKVVGCEVTAILIL 427

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Si_GI-514815267.pro YGLPRLLTGSI LAHEMMHAYLR LK-----GYRTLSPEV 406

Bd_GI-357157184.pro YGLPRLQTGSI LAHEMMHAYLR LK-----GYRSLSPQV 388

Br_DA1b.pro FGLPRLLTGSI LAHEMMHAWMLR LK-----GFRPLSQDV 418

Br_DA1a.pro FGLPRLLTGSI LAHEMMHAWMLR LK-----GFRTLSQDV 438

At_GI-15221983.pro FGLPRLLTGSI LAHEMMHAWMLR LK-----GFRTLSQDV 437

Tc_GI-508722773.pro YGLPRLLTGSI LAHEMMHAWMLR LQ-----GFRTLSQDV 383

Gm_GI-356564241.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 378

Gm_GI-356552145.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 382

Vv_GI-302142429.pro YGLPRLLTGSI LAHEMMHAWMLR LN-----GYRTLAQDV 385

Vv_GI-359492104.pro YGLPRLLTGSI LAHEMMHAWMLR LN-----GYRTLAQDV 388

Sl_GI-460385048.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 391

Os_GI-218197709.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 781

Os_GI-115466772.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 389

Bd_GI-357160893.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 415

Bd_GI-357164660.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 397

Sb_GI-242092232.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 389

Zm_GI-212275448.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 411

At_GI-240256211.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYPNLRPEV 456

At_GI-145360806.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GFRNLRPEV 427

At_GI-22326876.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRNLNMVL 1507

At_GI-30698242.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYKNLKLLEL 337

At_GI-30698240.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GHMNLNNIL 603

At_GI-15240018.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GHRNLNNIL 545

At_GI-334188680.pro YGLPRLLTGSI LAHEMMHAWMLR LK-----GYRTLSQDV 487

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Si_GI-514815267.pro EEGICQVLAHLWLESEITSGSGSMATTS AASSS-----SSTS--SSSKKA-KTEFEKRL 458

Bd_GI-357157184.pro EEGICQVLSHMLWLESEIIAGASGNTASTSV PSS-----SSAP--TSSSKKA-KTEFEKRL 440

Br_DA1b.pro EEGICQVMAHKWLEAE LAAGSRNSNAASSSSSS-----Y-----GGVKKGP-RSQYERKL 467

Br_DA1a.pro EEGICQVMAHKWLEAE LAAGSRNSNVASSSSSS-----RCVKKGP-RSQYERKL 485

At_GI-15221983.pro EEGICQVMAHKWLEAE LAAGSTNSNAASSSSSS-----QGLKKGP-RSQYERKL 485

Tc_GI-508722773.pro EEGICQVLAHMLWLTQLEYAS-SSNVASASSS-----S-----SRLQKGP-RPQFEKRL 431

Gm_GI-356564241.pro EEGICQVLAHMLWLESELSSASGSNFVSASSSSS-----S-----HTSRKGP-RPQFEKRL 427

Gm_GI-356552145.pro EEGICQVLSHMLWLESELSSASGSNFVSASSSSS-----S-----HTSRKGP-RPQFEKRL 431

Vv_GI-302142429.pro EEGICQVLAYMWLDAELTSGSGR-----SQCKERKL 415

Vv_GI-359492104.pro EEGICQVLAYMWLDAELTSGSGSNV-PSTSSAS-----TSSSKKA-GSQCKERKL 435

Sl_GI-460385048.pro EEGICQVLAHMLWLTQIASISSNGGASTSSGM-----SSSKQGI-RSPFERKL 439

Os_GI-218197709.pro EEGICQVLAHMLWIESEIIAGSGSNGASTSSSSS-----AS-----TSSSKKG-RSQFERKL 831

Os_GI-115466772.pro EEGICQVLAHMLWIESEIIAGSGSNGASTSSSSS-----AS-----TSSSKKG-RSQFERKL 439

Bd_GI-357160893.pro EEGICQVLAHMLWIESEIAGSGSNAASTSSSSS-----SS-----ISSKKG-RSQFERKL 465

Bd_GI-357164660.pro EEGICQVLAHMLWIESEIAGSGSNAASTSSSST-----S-----SKKG-RSQFERKL 444

Sb_GI-242092232.pro EEGICQVLAHLWIESEIAGSGSGAASSSSSGSS-----SS-----MSSKKG-RSQFEHKL 439

Zm_GI-212275448.pro EEGICQVLAHMLWIESEIAGSGSGSAASSSSSGSS-----SS-----TSSSKKG-RSQFEHRL 461

At_GI-240256211.pro EEGICQVLAHMLWLESETYAGSTLVDIASSSSS-----VVS-----ASSKKG-RSDFEKL 507

At_GI-145360806.pro EEGICQVLSYMWLESEVLSDPSTRNLPTSSVA-----TSSSSSFSNKKKG-KSNVEKRL 481

At_GI-22326876.pro EEGICQVLSYMWLESCQTYVFD-----TATIASSS-----SSRTPLSTTSKKVD-PSDFEKL 1560

At_GI-30698242.pro EEGICQVLAHMLWLESEIAGSGSNGASTSSSSS-----SSRTPLSTTSKKVD-PSDFEKL 397

At_GI-30698240.pro EEGICQVLAHMLWLESEIAGSGSNGASTSSSSS-----SSRTPLSTTSKKVD-PSDFEKL 659

At_GI-15240018.pro EEGICQVLAHMLWLESEIAGSGSNGASTSSSSS-----SSRTPLSTTSKKVD-PSDFEKL 601

At_GI-334188680.pro EEGICQVLAHMLWLESEIAGSGSNGASTSSSSS-----SSRTPLSTTSKKVD-PSDFEKL 538

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Si_GI-514815267.pro GEFFKHQIETDPSVAYGDGFRAGMRAVERYG--LRSTLDHKLITGSFP----- 504

Bd_GI-357157184.pro GAFIKNQIETDSSVEYGDGFRAGNRAVERYG--LRSTLDHMKITGSFP----- 487

Br_DA1b.pro GEFFKHQIESDASVPYGDGFRAGRLAVNKY--LWRTLEHIQMTGRFPV----- 514

Br_DA1a.pro GEFFKHQIESDASVPYGDGFRAGRLAVNKY--LPKLEHIQMTGRFPV----- 532

	At_GI-15221983.pro	GEFFKHQIESDASPVYGDGFRAGRLAVHKYG--LRKTLLEHIQMTGRFPV----	532
	Tc_GI-508722773.pro	GEFFKHQIESDTSPVYGDGFRAGHQAVYKYG--LRRTLEHIRMTGRFPY----	478
	Gm_GI-356564241.pro	GEFFKHQIESDISPVYGDGFRAGQKAVRKYG--LQRTLHHIRMTGTFPY----	474
	Gm_GI-356552145.pro	GEFFKHQIESDISPVYGGGFRAGQKAVSKYG--LQRTLHHIRMTGTFPY----	478
5	Vv_GI-302142429.pro	GQFFKHQIESDTSLVYGAGFRAGHQAVLKYG--LPATLKHIHLTGNEFPY----	462
	Vv_GI-359492104.pro	GQFFKHQIESDTSLVYGAGFRAGHQAVLKYG--LPATLKHIHLTGNEFPY----	482
	Sl_GI-460385048.pro	GDFFKHQIESDTSPIYGNNGFRAGNQAVLKYG--LERTLDHIRMTGTFPY----	486
	Os_GI-218197709.pro	GDFFKHQIESDTSMAYGDGFRAGNRAVLQYG--LKRTLEHIRLTGTFFP----	878
	Os_GI-115466772.pro	GDFFKHQIESDTSMAYGDGFRAGNRAVLQYG--LKRTLEHIRLTGTFFP----	486
10	Bd_GI-357160893.pro	GDFFKHQIESDTSVAYGNNGFRSGNQAVLQYG--LKRTLEHIWLTGTWFP----	512
	Bd_GI-357164660.pro	GDFFKHQIESDTSVAYGDGFRAGNQAVLQYG--LKRTLEHIRLTGTLPF----	491
	Sb_GI-242092232.pro	GDFFKHQIETDTSMAYGEGFRAGNRAVLQYG--LKRTLEHIRLTGTFFP----	486
	Zm_GI-212275448.pro	GDFFKHQIETDTSMAYGDGFRAGNRAVLHYG--LKRTLEHIRLTGTFFP----	508
15	At_GI-240256211.pro	GEFFKHQIESDSSAYGDGFRQGNQAVLKHG--LRRTLDHIRLTGTFFP----	553
	At_GI-145360806.pro	GEFFKHQIAHDASPAYGGGFRANAAACKYG--LRRTLDHIRLTGTFFP----	528
	At_GI-22326876.pro	VNFCKHQIETDESPFFGDGFRKVNKMMASSNNHSLKDTLKEIISISKIPQYSKL	1613
	At_GI-30698242.pro	VEFCMNQIKEDDSPVYGLGFKQVYEMMVSNYNINIKDTLKDIVSASNATPDSTV	450
	At_GI-30698240.pro	VEFCKNQIETDESPVYGLGFRTVNEMVTNS--SLQETLKEILRR-----	702
	At_GI-15240018.pro	VEFCKNQIETDDSPVYGLGFRTVNEMVTNS--SLQETLKEILRQR-----	644
20	At_GI-334188680.pro	VEFCKDQIETDDSPVYGVGFRKVNQMVSDS--SLHKILKSIQHWTKPDSNL--	587
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Table 3 Alignment of DA1 proteins (SEQ ID NOS: 41-64)

Zm_GI-223973923.pro -----MNSS--RQMEHLYINTGFPYTITESFMDFFEGLYAHA 36
 Sb_GI-242042045.pro -----MNSC--RQMEHLYINTGFPYTITESFMDFFEGLYAHA 36
 Zm_GI-226496789.pro -----MTSS--RQMEHLYINTGFPYTITESFMDFFEGLYAHA 36
 5 Os_GI-222624282.pro MTESHERDTEVTRWQVHDPSEGMNGS--RQMEHLYINTGFPYTITESFMDFFEGLYAHA 58
 Os_GI-115451045.pro -----MNGS--RQMEHLYINTGFPYTITESFMDFFEGLYAHA 36
 Bd_GI-357113826.pro -----MNGS--RQMEHLYINTGFPYTITESFMDFFEGLYAHA 36
 Sl_GI-460410949.pro -----MNWN--QQTEIYYTNGAMPYNSIGSFMDFFGGVTYDHY 36
 10 Rc_GI-255582236.pro -----MEVHYINTGFPYTVTESFLDFFEGLSHVPV 30
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 25 Os_GI-115451045.pro DFAIADAFHDQ--ANPYWAMMHTNSYKYGYSAG--NYYSYGHVYDMDYMRADGGRI 92
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	Sl_GI-460370551.pro	QINFPCCKHIYHTECGSKWLSINKRCSLM	NEVVQW-----	243
		*: : *. * * * : : * * * * *: *		

40

Table 4 Alignment of EOD1 proteins (SEQ ID NOS: 74-90)

Claims:

1. A method of increasing the yield of a plant comprising;
reducing the expression or activity of an DA2 polypeptide
5 within cells of said plant,
wherein the plant has reduced DA1 and/or EOD1 expression or
activity.
2. A method according to claim 1 wherein the plant has reduced
10 EOD1 activity.
3. A method according to claim 1 or claim 2 wherein the plant has
reduced DA1 expression or activity
- 15 4. A method according to claim 3 wherein the plant expresses a
dominant negative DA1 polypeptide.
5. A method according to any one of claims 1 to 4 wherein the
expression or activity of the DA2 polypeptide is abolished in the
20 cells of the plant.
6. A method of increasing the yield of a plant comprising;
reducing the expression or activity of a DA2 polypeptide, and;
reducing the expression or activity of a DA1 polypeptide
25 and/or a EOD polypeptide within cells of said plant.
7. A method according to claim 6 wherein the method comprises
reducing EOD1 polypeptide expression or activity within cells of
said plant.
30
8. A method according to claim 7 wherein the expression or
activity of the EOD1 polypeptide is reduced by introducing a
mutation into the nucleotide sequence of the plant cell which
encodes the EOD1 polypeptide or which regulates its expression and
35 regenerating the plant from the mutated cell.
9. A method according to claim 7 wherein the expression or
activity of the EOD1 polypeptide is reduced by incorporating a
heterologous nucleic acid which expresses a suppressor nucleic acid
40 which reduces expression of the EOD1 polypeptide into said plant
cell.

10. A method according to any one of claims 6 to 9 wherein the method comprises reducing DA1 polypeptide expression or activity within cells of said plant.

11. A method according to claim 10 wherein the expression or activity of the DA1 polypeptide is reduced by introducing a mutation into the nucleotide sequence of the plant cell which encodes the DA1 polypeptide or which regulates its expression and regenerating the plant from the mutated cell.

12. A method according to claim 10 wherein the expression or activity of the DA1 polypeptide is reduced by incorporating a heterologous nucleic acid which expresses a suppressor nucleic acid which reduces expression of the DA1 polypeptide into said plant cell.

13. A method according to claim 10 wherein the expression or activity of the DA1 polypeptide is reduced by expressing a dominant negative DA1 polypeptide within cells of said plant.

14. A method according to any one of claims 6 to 13 wherein the expression or activity of the DA2 polypeptide is reduced by introducing a mutation into the nucleotide sequence of the plant cell which encodes the DA2 polypeptide or which regulates its expression and regenerating the plant from the mutated cell.

15. A method according to any one of claims 6 to 13 wherein the expression or activity of the DA2 polypeptide is reduced by incorporating a heterologous nucleic acid which expresses a suppressor nucleic acid which reduces expression of the DA2 polypeptide into said plant cell.

16. A method according to any one of claims 6 to 16 wherein the expression or activity of the DA2 polypeptide is abolished in the cells of the plant.

17. A method of producing a plant with an increased yield comprising:

providing a plant cell that is deficient in the expression or activity of DA1, EOD1 or both DA1 and EOD1,

incorporating a heterologous nucleic acid which reduces the expression or activity of a DA2 polypeptide; or introducing a

mutation which reduces the expression or activity of a DA2 polypeptide into the plant cell, and;
regenerating the plant from one or more transformed cells.

5 18. A method according to claim 17 wherein the plant cell is deficient in EOD1 polypeptide expression or activity.

19. A method according to claim 18 wherein the plant cell comprises a mutation into the nucleotide sequence of the plant cell
10 which encodes the EOD1 polypeptide or which regulates its expression or a further heterologous nucleic acid which expresses a suppressor nucleic acid which reduces expression of the EOD1 polypeptide in said plant cell.

15 20. A method according to any one of claims 17 to 19 wherein the plant cell is deficient in DA1 polypeptide expression or activity.

21. A method according to claim 20 wherein the plant cell comprises a mutation into the nucleotide sequence of the plant cell
20 which encodes the DA1 polypeptide or which regulates its expression; a further heterologous nucleic acid which expresses a suppressor nucleic acid which reduces expression of the DA1 polypeptide in said plant cell; or a further heterologous nucleic acid that expresses a dominant negative DA1 polypeptide within cells of said plant.

25 22. A method according to any one of claims 17 to 21 wherein the heterologous nucleic acid expresses a suppressor nucleic acid which reduces expression of the DA2 polypeptide into said plant cell.

30 23. A method according to any one of claims 17 to 22 wherein the heterologous nucleic acid abolishes the expression or activity of a DA2 polypeptide in the cell of the plant.

24. A method according to any one of the preceding claims wherein
35 the plant has increased plant size, seed size and/or organ size relative to wild type plants.

25. A method according to any one of the preceding claims wherein the nucleic acid encoding the dominant negative DA polypeptide
40 and/or the suppressor nucleic acid is operably linked to a heterologous promoter.

26. A method according to claim 25 wherein the promoter is a tissue-specific promoter.

27. A method according to claim 26 wherein the promoter is an inducible promoter.

28. A method according to any one of the preceding claims wherein the heterologous nucleic acid is comprised in one or more vectors.

29. A method according to any one of claims 1 to 15 comprising sexually or asexually propagating or growing off-spring or descendants of the plant having reduced DA2 expression or activity.

30. A method according to any one of the preceding claims wherein the DA2 polypeptide comprises a RING domain of SEQ ID NO: 1.

31. A method according to claim 30 wherein the DA2 polypeptide comprises a RING domain of SEQ ID NO:2.

32. A method according to claim 31 wherein the DA2 polypeptide comprises a first consensus domain of SEQ ID NO: 36.

33. A method according to claim 31 or claim 32 wherein the DA2 polypeptide comprises a second consensus domain of SEQ ID NO: 37.

34. A method according to any one of claims 30 to 33 wherein the DA2 polypeptide comprises an amino acid sequence having at least 20% sequence identity to any one of SEQ ID NOS: 20 to 35.

35. A method according to any one of the preceding claims wherein the DA1 polypeptide comprises a UIM1 domain of SEQ ID NO: 68 and a UIM2 domain of SEQ ID NO: 69.

36. A method according to claim 35 wherein the DA1 polypeptide comprises a LIM domain of SEQ ID NO: 38 or 39.

37. A method according to claim 35 or claim 36 wherein the DA1 polypeptide comprises a C terminal region having at least 20% sequence identity to residues 229 to 532 of SEQ ID NO: 45.

38. A method according to any one of claims 35 to 37 wherein the DA1 protein comprises a sequence having at least 20% sequence identity to any one of SEQ ID NOS: 41 to 64.

39. A method according to any one of the preceding claims wherein the dominant negative DA1 comprises an R to K substitution at the position in the amino acid sequence of the DA1 polypeptide that is equivalent to position 358 of the DA1 polypeptide of SEQ ID NO: 45.

40. A method according to any one of the preceding claims wherein the EOD1 polypeptide comprises a sequence having at least 20% sequence identity to any one of SEQ ID NOS: 74 to 90.

41. A method according to any one of claims 1 to 40 wherein the plant is a higher plant.

42. A method according to claim 41 wherein the plant is an agricultural plant selected from the group consisting of *Lithospermum erythrorhizon*, *Taxus* spp, tobacco, cucurbits, carrot, vegetable brassica, melons, capsicums, grape vines, lettuce, strawberry, oilseed brassica, sugar beet, wheat, barley, maize, rice, soyabeans, peas, sorghum, sunflower, tomato, potato, pepper, chrysanthemum, carnation, linseed, hemp and rye.

43. A plant having reduced expression or activity of a DA2 polypeptide and reduced expression or activity of a DA1 polypeptide and/or a EOD1 polypeptide,

wherein the expression or activity of one or more of said DA2, DA1 and EOD1 polypeptides is reduced by the incorporation of a heterologous nucleic acid into one or more cells of the plant.

44. A plant according to claim 43 which produced by a method according to any one of claims 1 to 42.

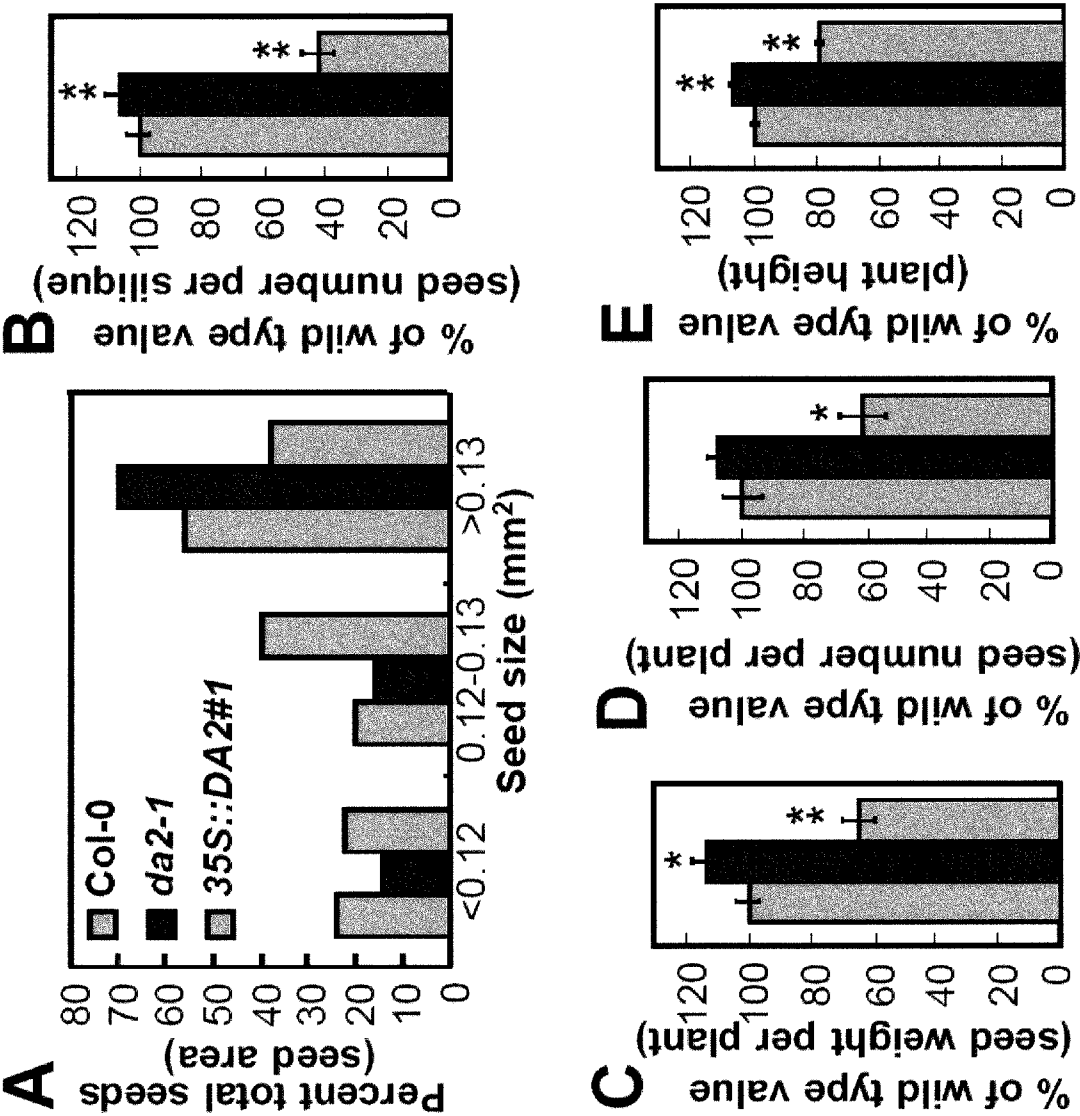


Figure 1

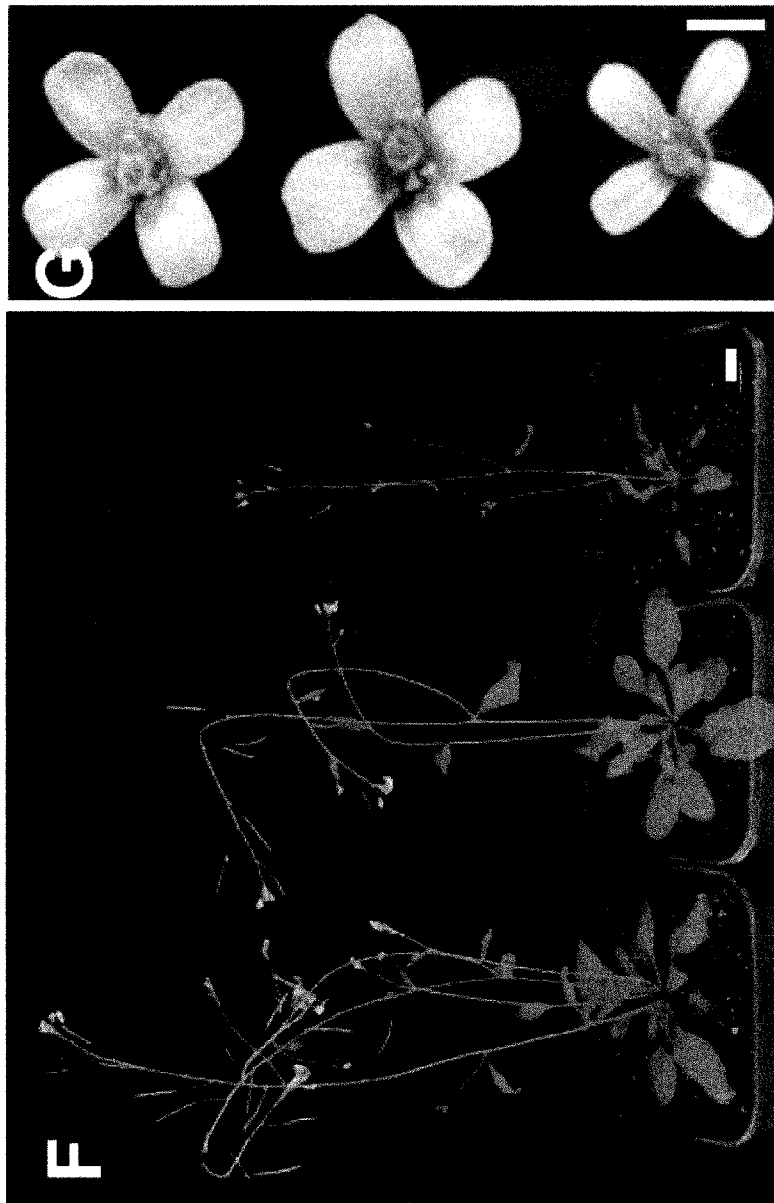


Figure 2

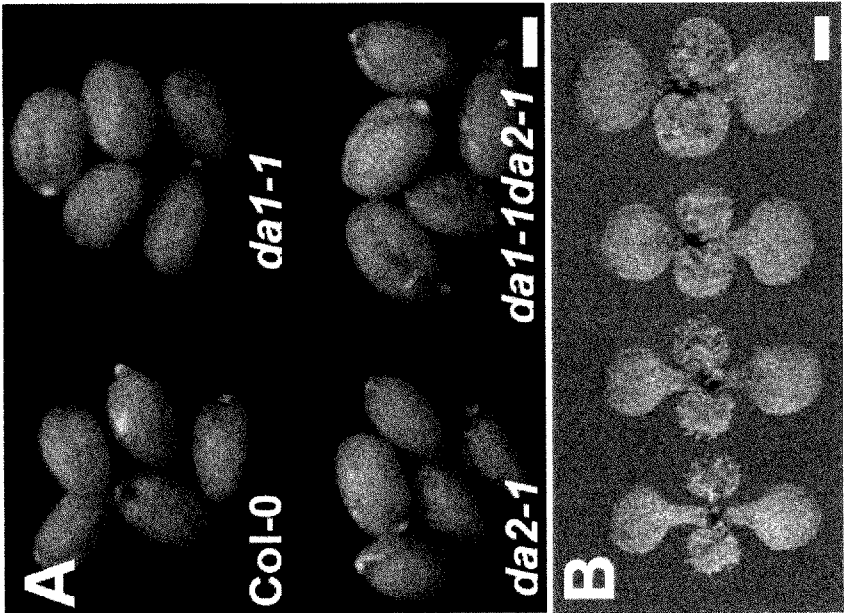
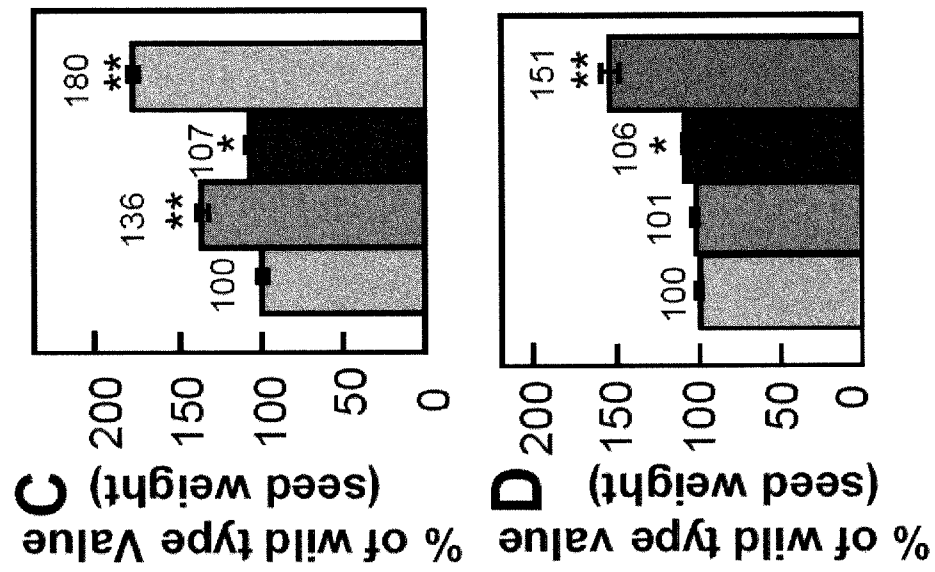


Figure 3

ANY REFERENCES TO FIGURES 3I AND 3J
SHALL BE CONSIDERED NON-EXISTENT

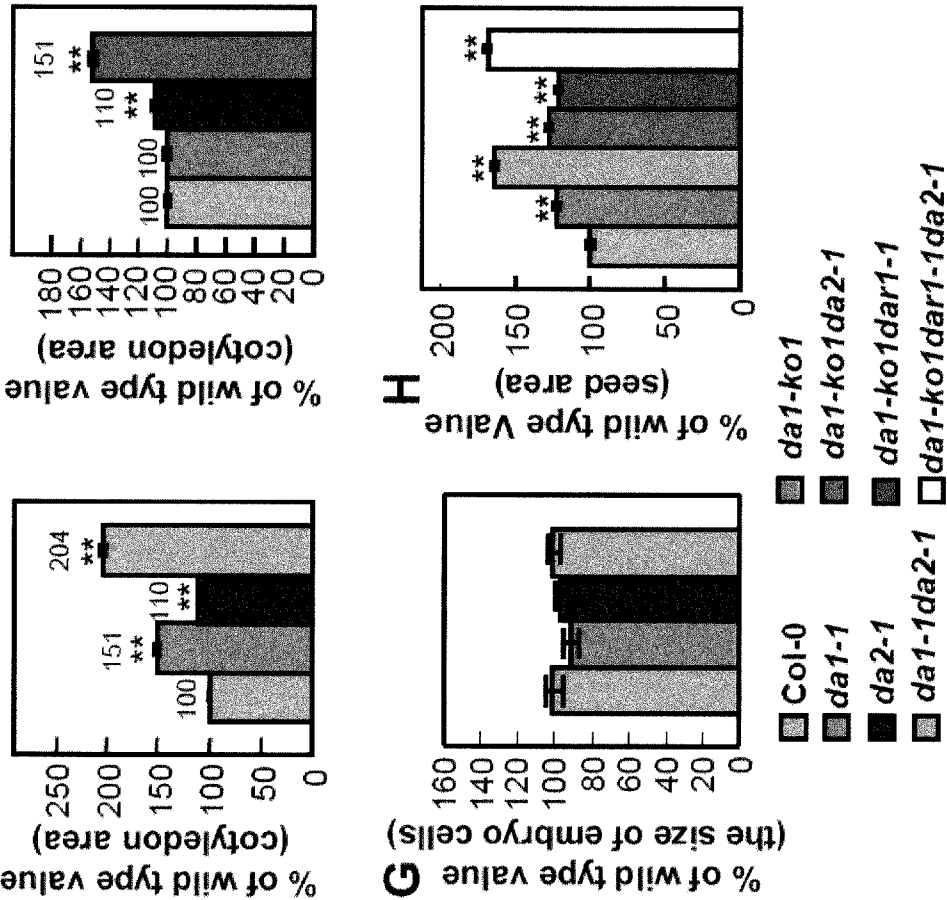


Figure 4

ANY REFERENCE TO FIGURE 4C SHALL BE
CONSIDERED NON-EXISTENT

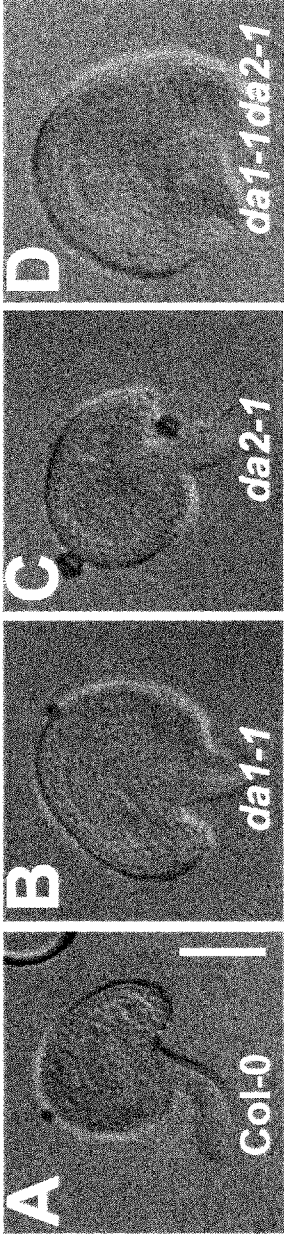


Figure 5

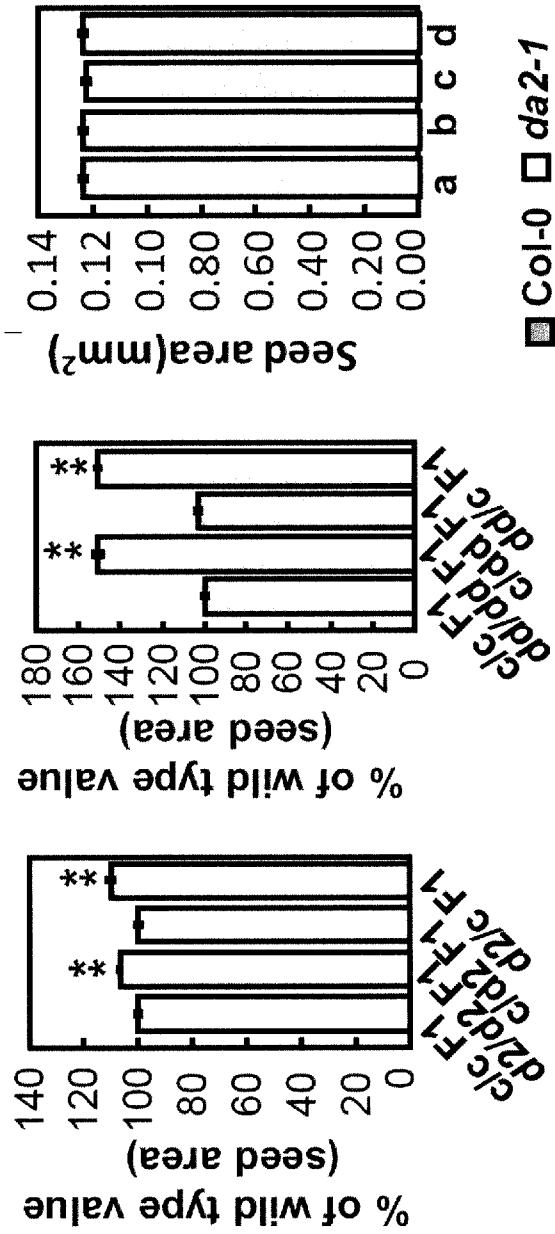


Figure 6

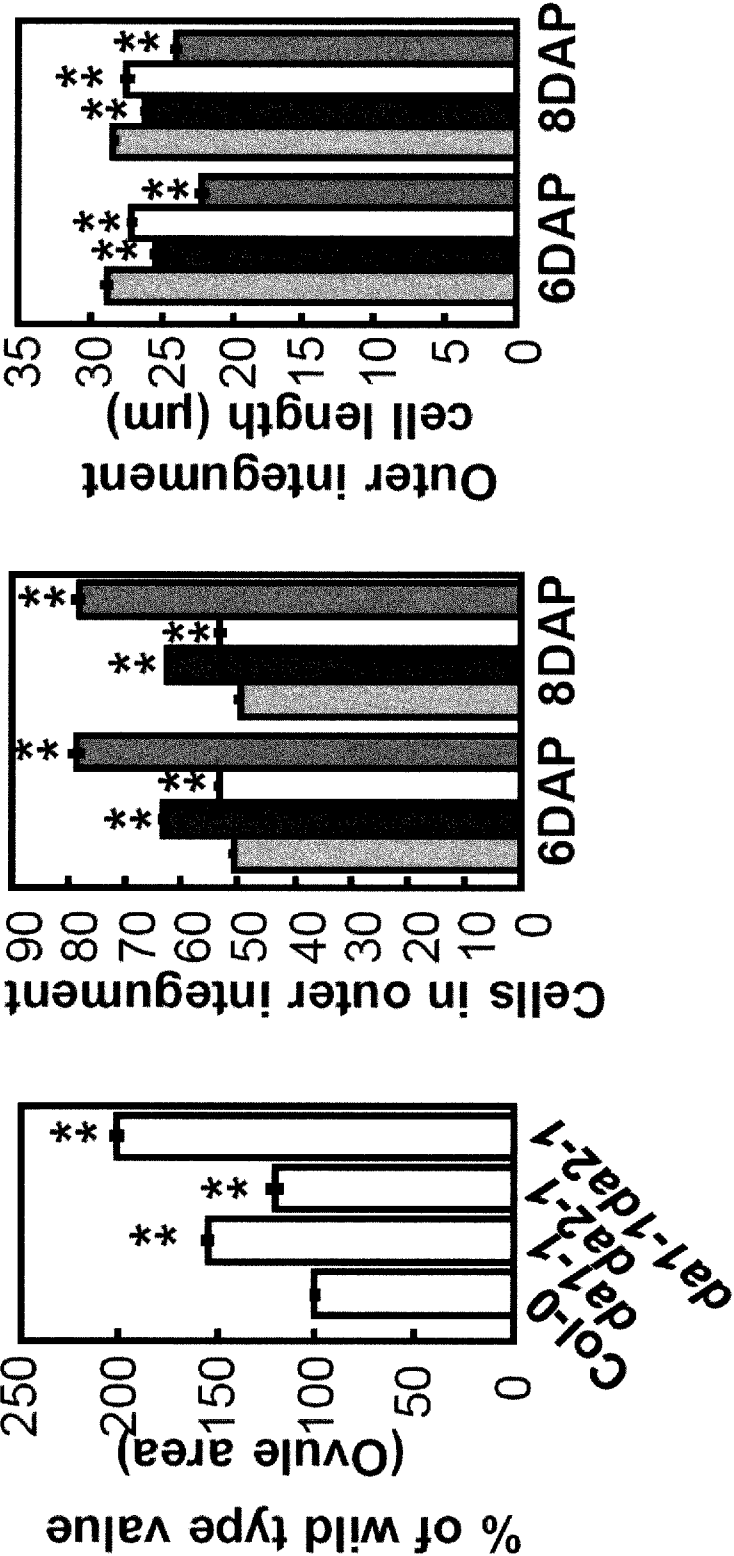


Figure 7

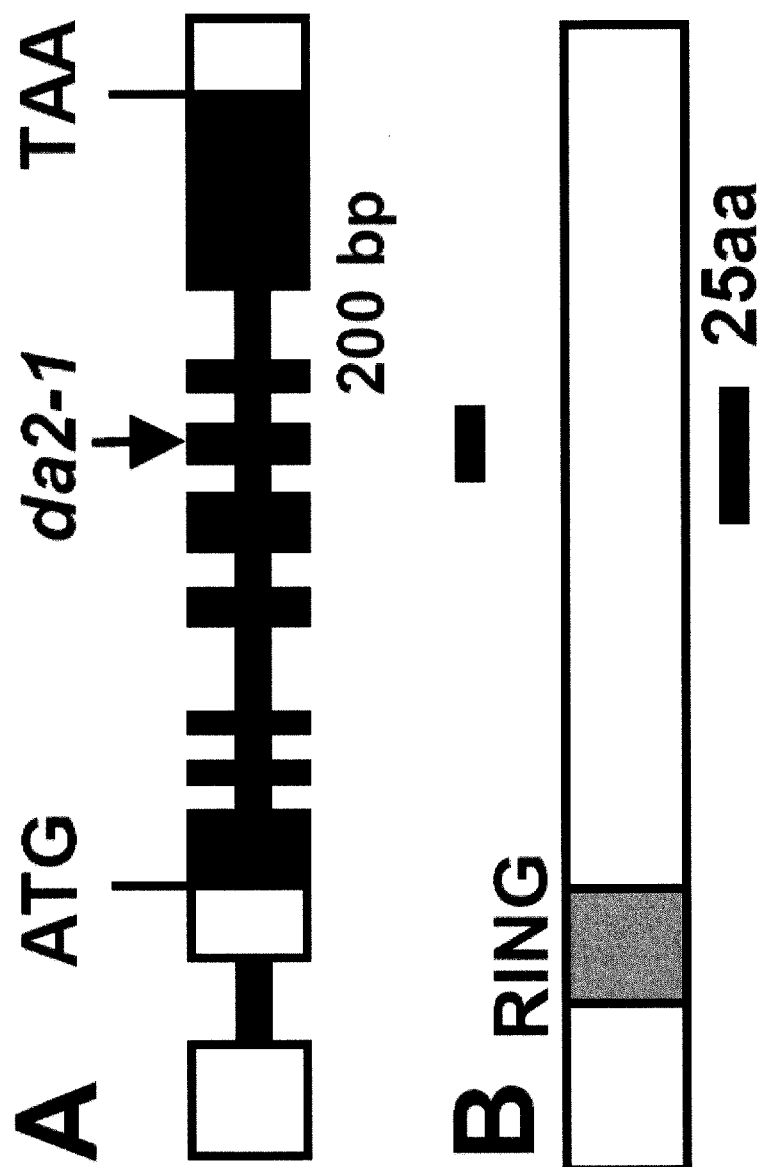


Figure 8

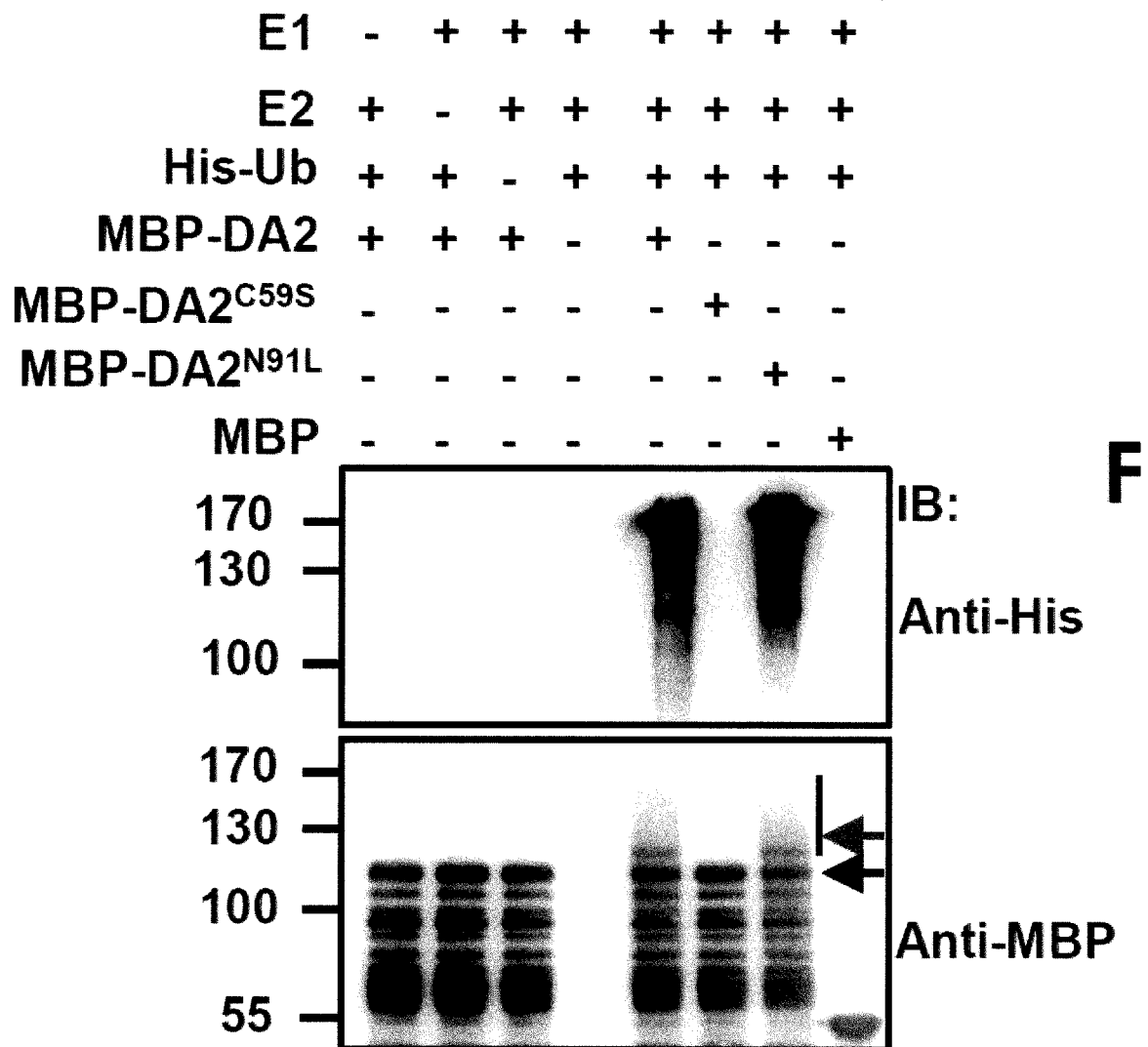


Figure 9

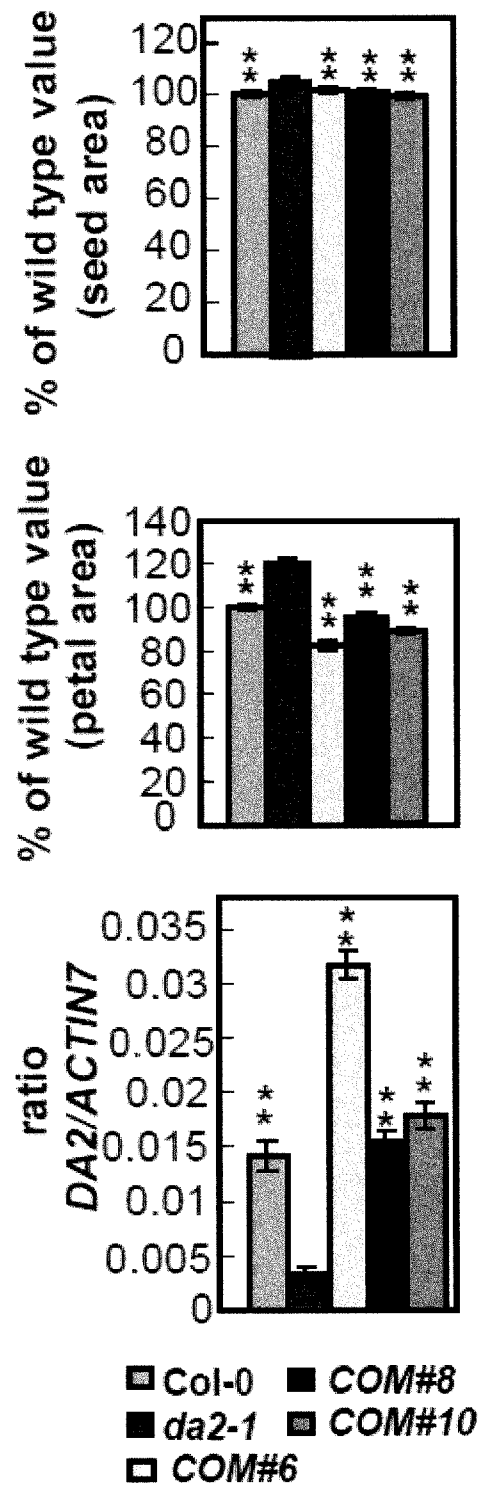


Figure 10

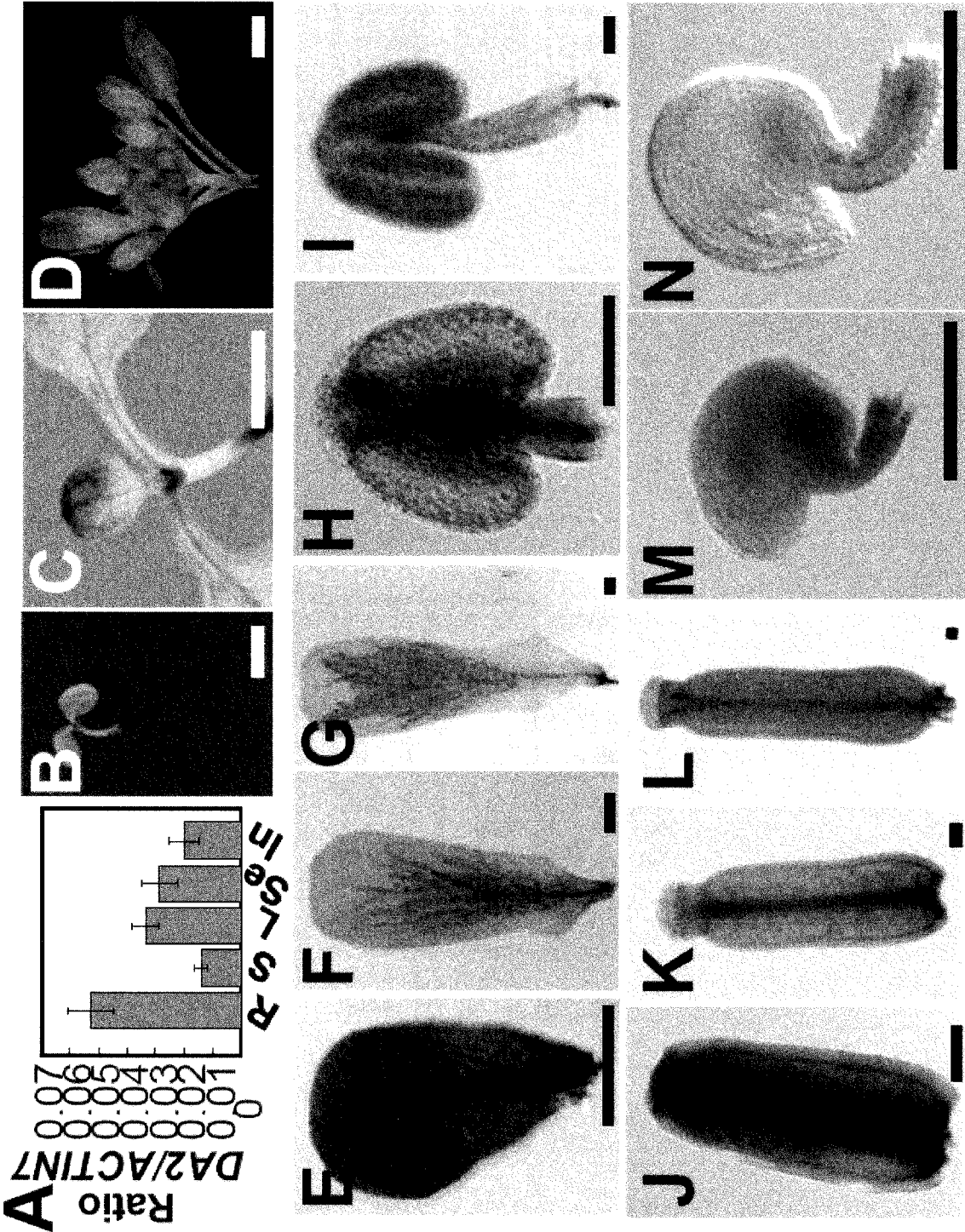


Figure 11

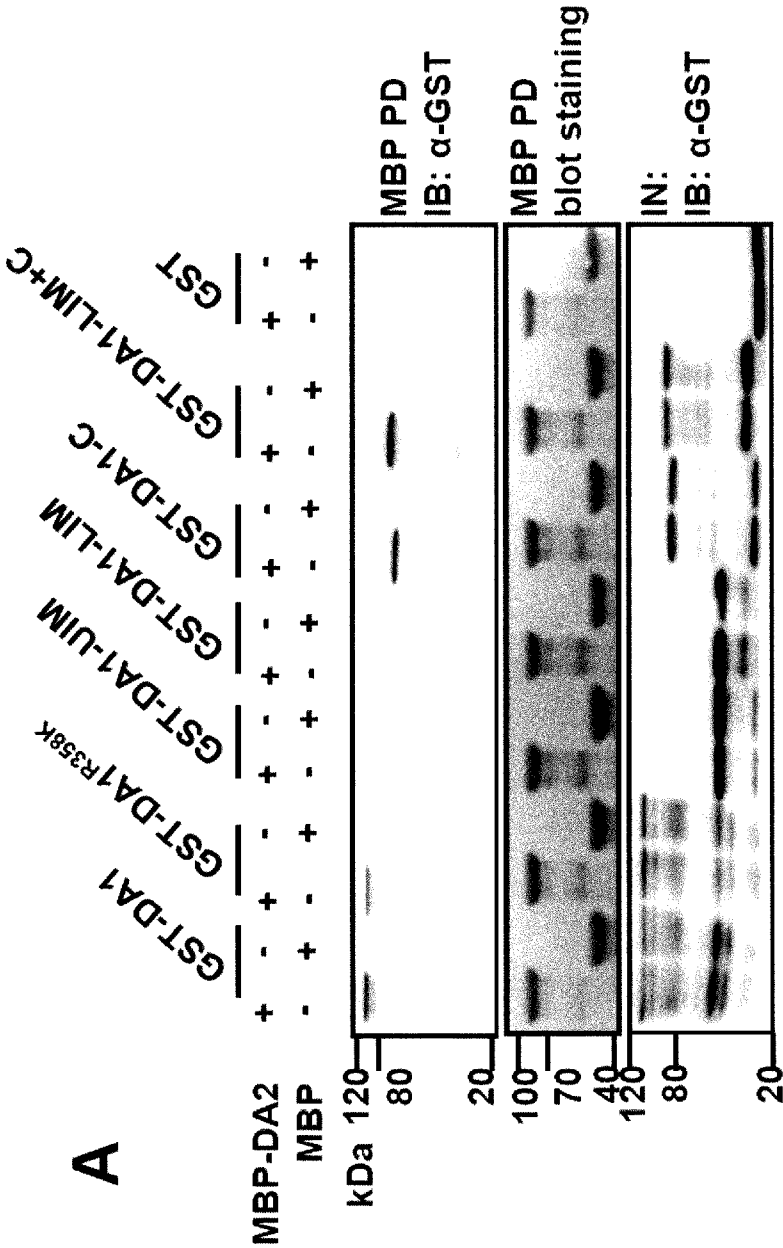


Figure 12

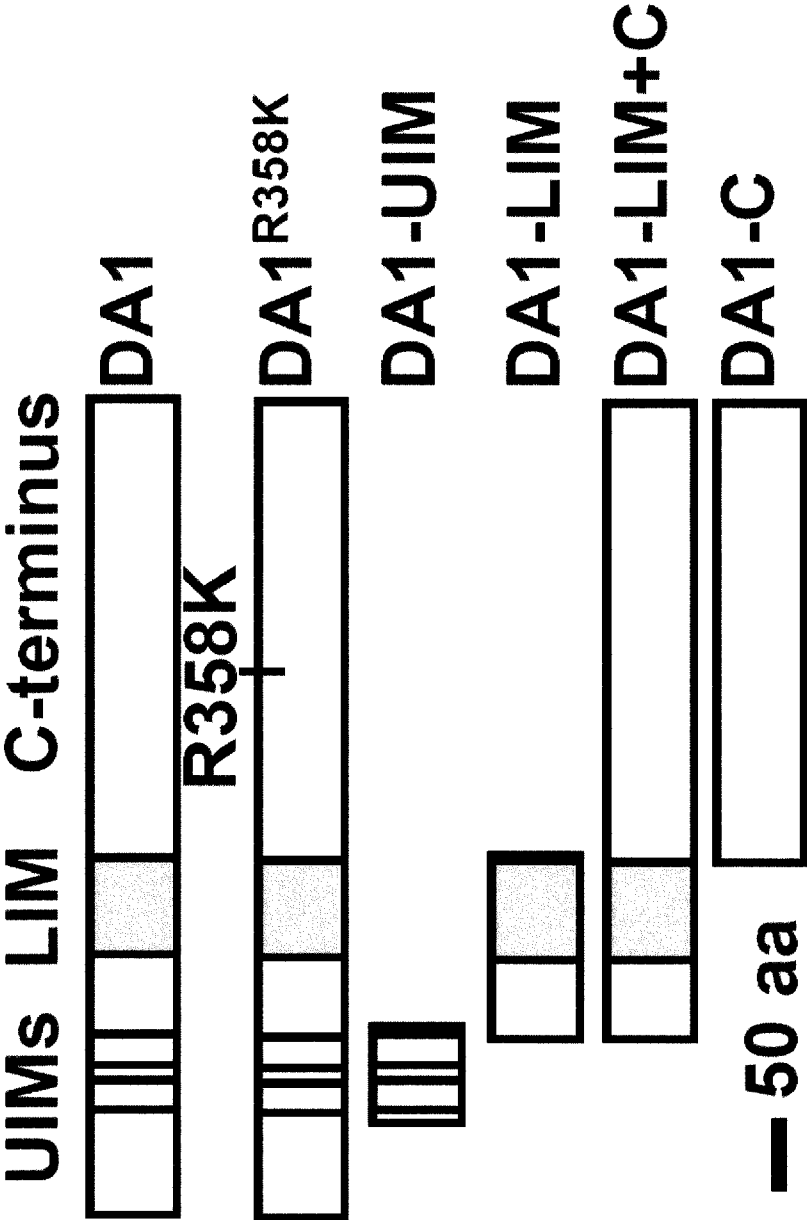


Figure 13

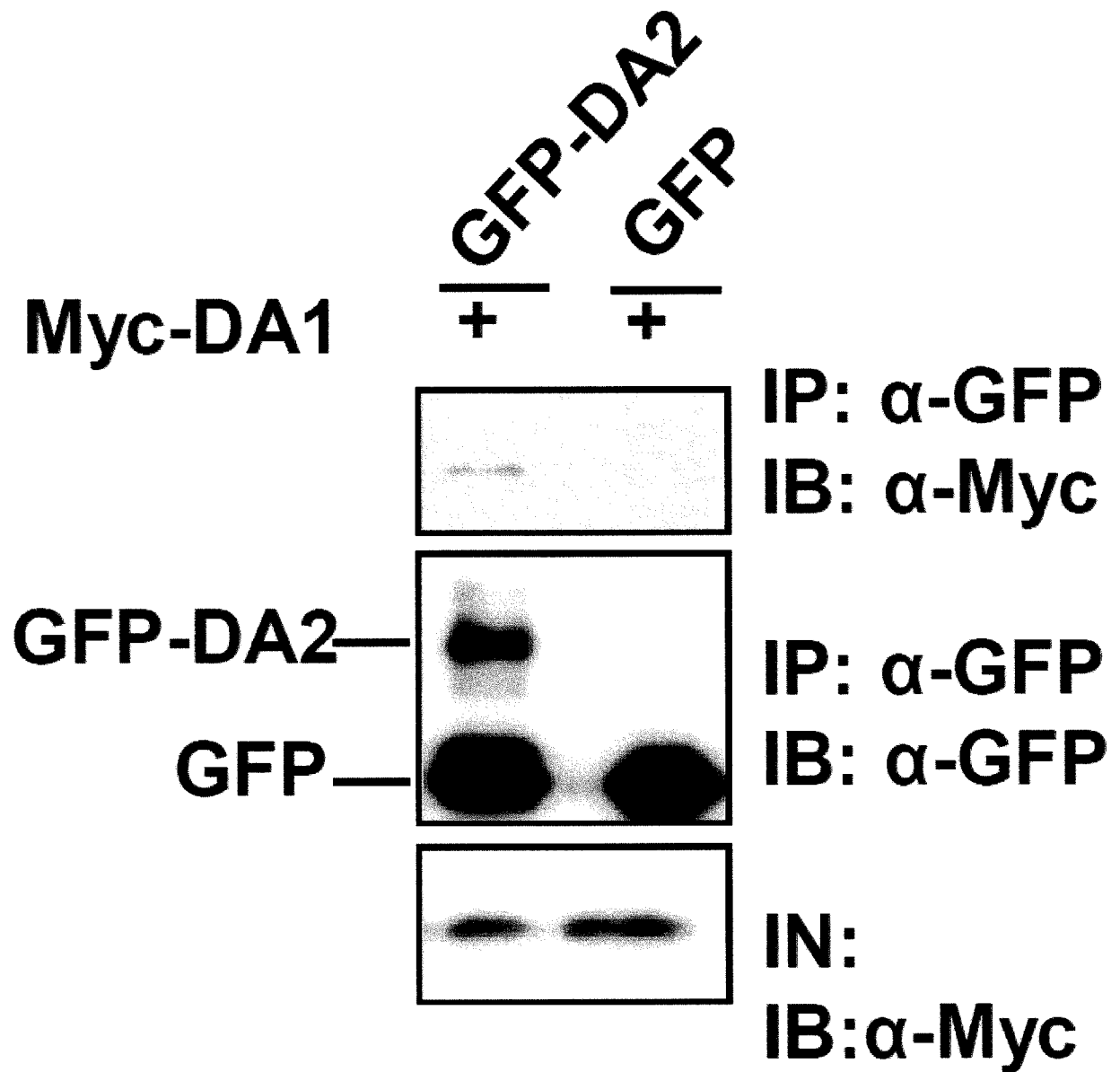


Figure 14

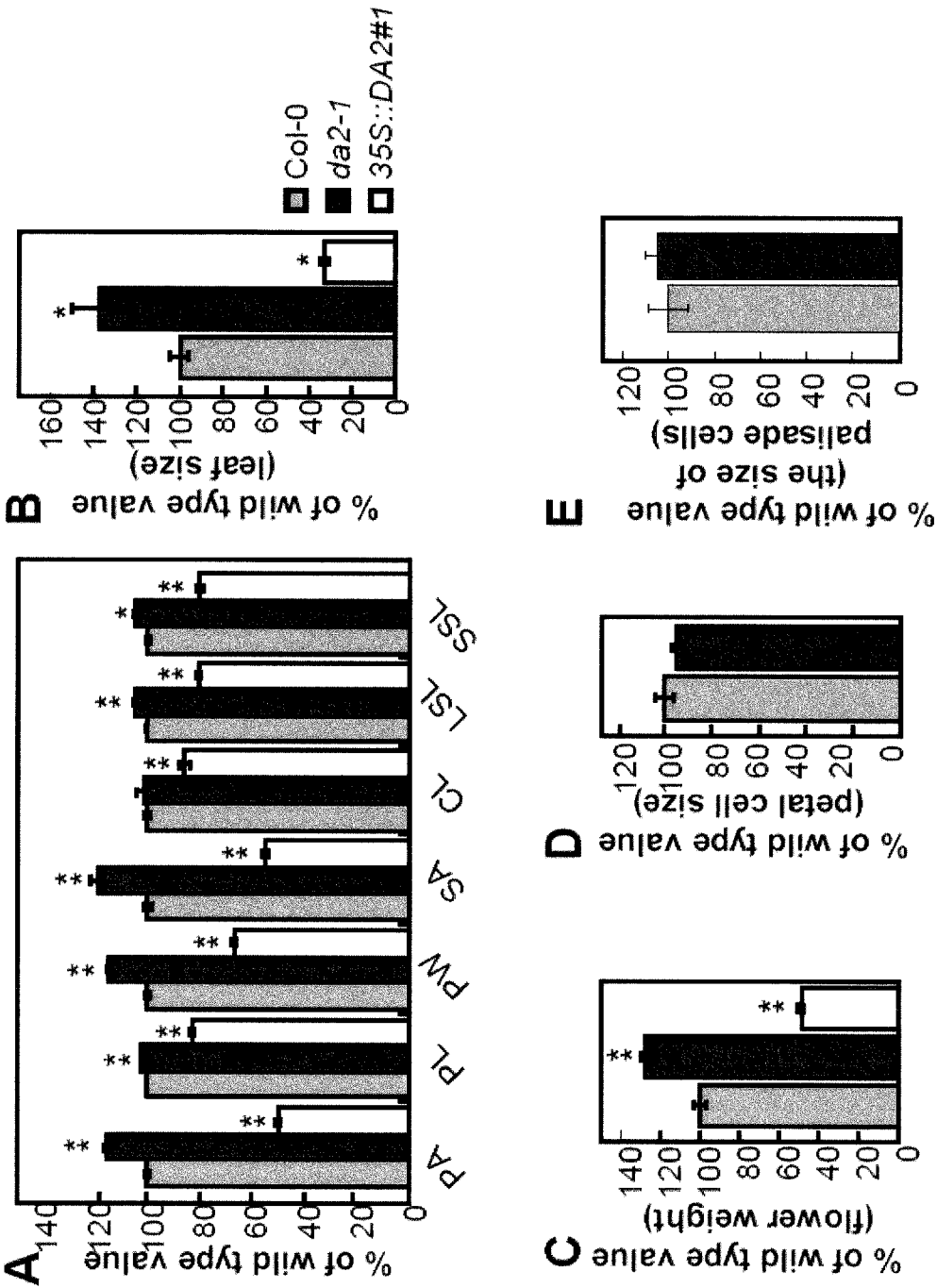


Figure 15

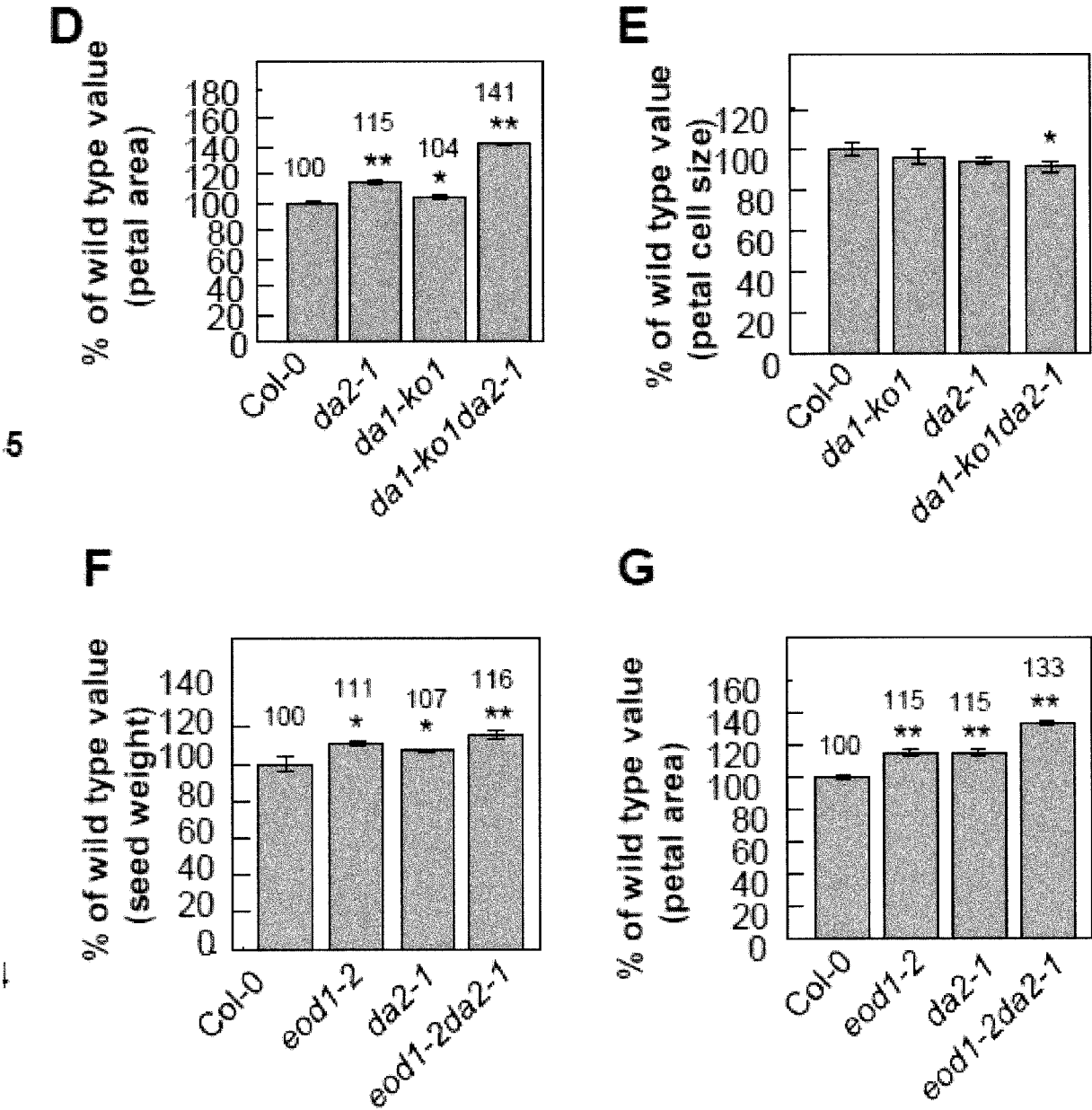


Figure 16

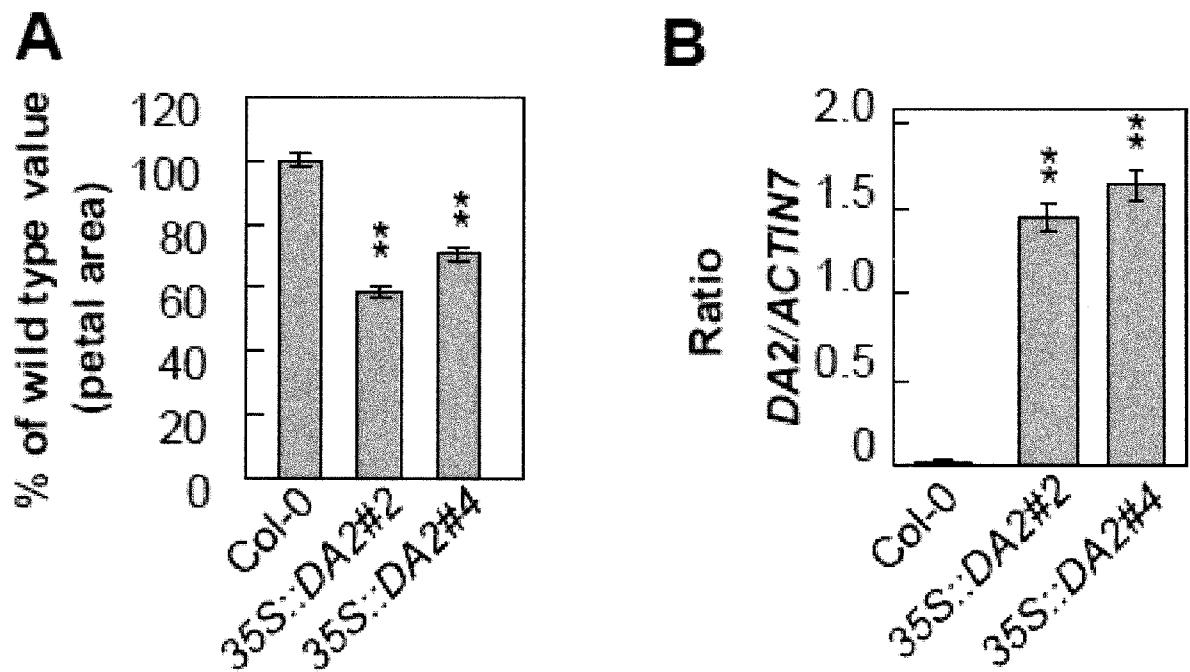


Figure 17

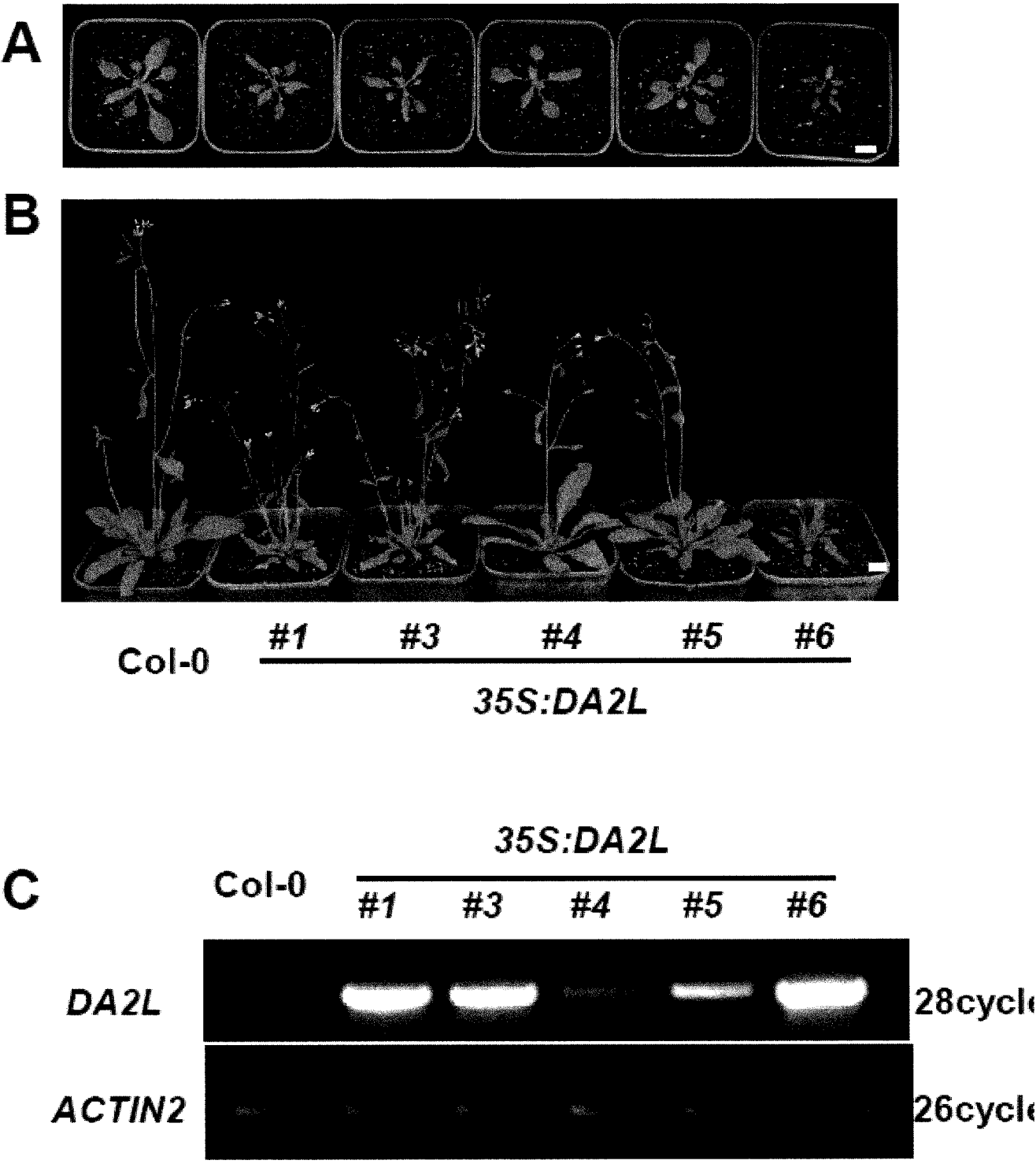


Figure 18

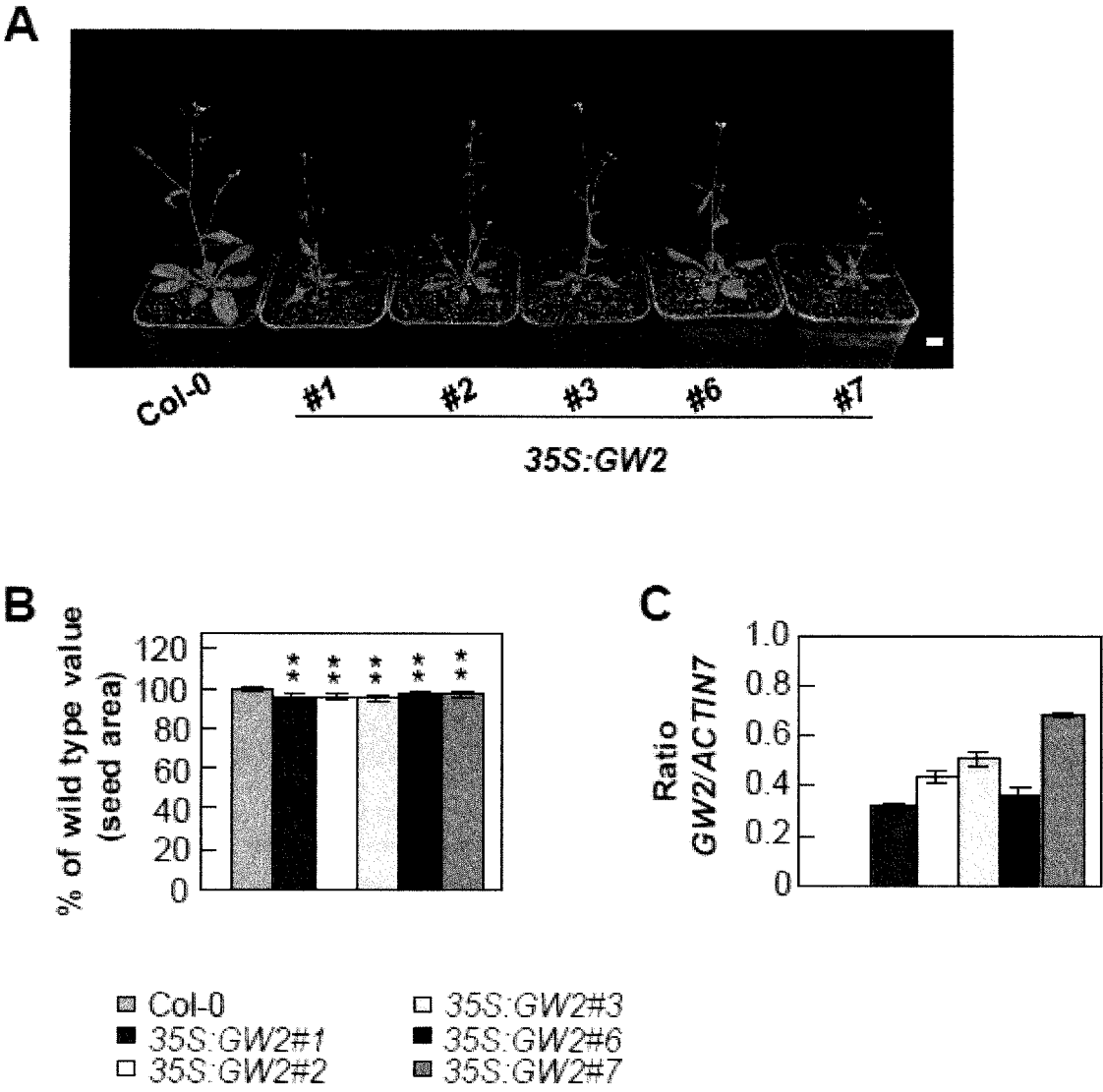


Figure 19

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/066427

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/415 A61K38/53 C12N15/82
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBL, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/047525 A1 (PLANT BIOSCIENCE LTD [GB]; BEVAN MICHAEL [GB]; LI YUNHAI [CN]) 16 April 2009 (2009-04-16)	1-29, 35-44
Y	claims 41-46 page 5, lines 14-15 page 2, line 35 - page 3, line 3 -----	30-34
X	KRIZEK ET AL: "Making bigger plants: key regulators of final organ size", CURRENT OPINION IN PLANT BIOLOGY, QUADRANT SUBSCRIPTION SERVICES, GB, vol. 12, no. 1, 1 February 2009 (2009-02-01), pages 17-22, XP025770802, ISSN: 1369-5266, DOI: 10.1016/J.PBI.2008.09.006 [retrieved on 2008-10-23] page 21, column 1, lines 23-27 ----- -/-	1-29, 35-44



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 October 2014

Date of mailing of the international search report

30/10/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Obel, Nicolai

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/066427

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