

***dwarf and delayed-flowering 1*, a novel *Arabidopsis* mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor**

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Summary

A novel gibberellin (GA)-deficient mutant designated *dwarf and delayed-flowering 1* (*ddf1*) was isolated from a library of activation-tagged *Arabidopsis*. This mutant showed dwarfism and late-flowering, but the phenotype was rescued by exogenous GA₃ like known mutants defective in GA biosynthesis. The contents of bioactive GA₄ and GA₁ were in fact decreased in *ddf1* at least partially through the repression of biosynthetic steps catalyzed by GA 20-oxidase (GA20ox). Genetic and molecular analyses revealed that the *ddf1* phenotypes are caused by increased or ectopic expression of a putative AP2 transcription factor. Overexpression of *DDF2*, encoding another putative AP2 transcription factor closely related to *DDF1*, also conferred the *ddf1*-like phenotype. Among genes encoding (putative) AP2 transcription factors in *Arabidopsis*, *DDFs* are phylogenetically close to dehydration-responsive element binding protein (*DREB1*)/C-repeat binding factor (*CBF*) genes, which are known to be involved in stress responses. The *ddf1* mutation upregulates a stress-related gene *RD29A*. *DDF1* mRNA is strongly induced by high-salinity stress within 1 h. Moreover, transgenic plants overexpressing *DDF1* showed increased tolerance to high-salinity stress. These results suggest that *DDF1* is involved in the regulation of GA biosynthesis and stress tolerance. The possible relation between the contents of endogenous GAs and acquisition of stress protection is discussed.

Keywords: *dwarf and delayed-flowering 1*, gibberellin, AP2 transcription factor, DREB1/CBF family, dwarfism, *Arabidopsis*.

Introduction

Gibberellins (GAs) are plant hormones that are involved in growth and development; they control seed germination, stem elongation, leaf expansion, trichome development, and flowering in *Arabidopsis*. GAs are formed from geranylgeranyl diphosphate via a multistep process. Recently, most genes for GA biosynthetic enzymes have been identified in *Arabidopsis* (Figure 1). Mutants deficient in GA biosynthesis, such as *ga1–ga5* (Koornneef and van der Veen, 1980), exhibit various phenotypes (dwarfism, dark-green leaves, late-flowering) that are rescued by exogenously applied GA.

The biosynthesis of GAs is regulated by both developmental and environmental stimuli (reviewed by Hedden and Phillips, 2000; Olszewski *et al.*, 2002; Yamaguchi and Kamiya, 2000). *Arabidopsis* *ent-copalyl* diphosphate synthase is tightly controlled by the developmental stage.

This gene is expressed in rapidly growing tissues and in the vascular elements of expanded leaves (Silverstone *et al.*, 1997). GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) are encoded by small gene families in *Arabidopsis*, and their transcripts accumulate differentially in tissues (Chiang *et al.*, 1995; Phillips *et al.*, 1995; Yamaguchi *et al.*, 1998). The levels of GAs are homeostatically modulated through negative feedback regulation of the expression of GA20ox (Xu *et al.*, 1995) and GA3ox genes (Chiang *et al.*, 1995; Cowling *et al.*, 1998), and through positive feedforward regulation of the gene for GA 2-oxidase (GA2ox), an enzyme that deactivates GA (Thomas *et al.*, 1999). Endogenous levels of other plant hormones such as auxin and brassinosteroids (BRs) might affect the expression of GA biosynthetic genes (Bouquin *et al.*, 2001; Ross *et al.*, 2000). Moreover, transcription levels of the GA20ox gene are regulated by the photoperiod

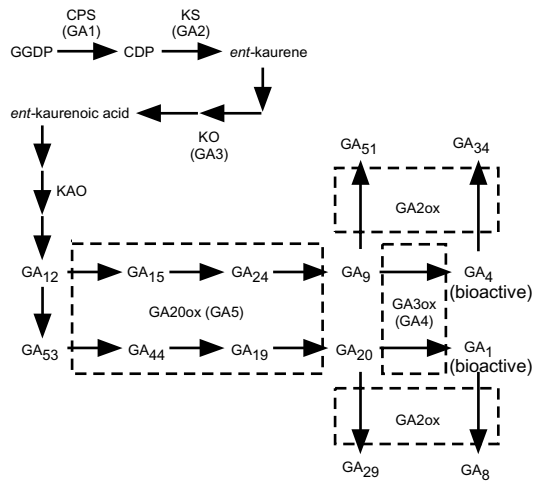


Figure 1. Pathways of GA synthesis and degradation in *Arabidopsis*. Geranylgeranyl diphosphate (GGDP), *ent*-copalyl diphosphate (CDP), *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KAO), *ent*-kaurenoic acid oxidase (KAO), GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox), GA 2-oxidase (GA2ox).

(Wu *et al.*, 1996; Xu *et al.*, 1997). GA3ox genes in germinating seeds are upregulated by red light, and reversed by far-red light through phytochrome B (Yamaguchi *et al.*, 1998). However, molecular mechanisms of the transcriptional regulation of these genes are largely unknown. Moreover, the possibility of the post-transcriptional regulation of GA biosynthetic enzymes remains to be evaluated.

To date, only two transcription factors involved in the regulation of GA biosynthetic genes have been identified. Tobacco *Nicotiana tabacum* homeobox 15 (NTH15) is a KNOX homeodomain protein that represses the expression of a GA20ox gene by binding directly to its first intron (Sakamoto *et al.*, 2001; Tanaka-Ueguchi *et al.*, 1998). Ectopic expression of NTH15 in transgenic plants reduced the levels of bioactive GAs and intermediates produced by GA20ox, leading to dwarfism with abnormal leaf and flower morphology. An ortholog of this gene, *Oryza sativa* homeobox 1 (OSH1), was found in rice (Kusaba *et al.*, 1998). Tobacco repression of shoot growth (RSG) is a basic leucine zipper protein (bZIP)-type transcription factor, which binds a sequence in the promoter region of the *ent*-kaurene oxidase gene and upregulates its expression (Fukazawa *et al.*, 2000). In *Arabidopsis*, no transcriptional regulators of GA biosynthesis have been identified so far.

In this paper, we report the isolation and characterization of a novel GA-deficient mutant designated *dwarf and delayed-flowering 1* (*ddf1*). We show that the *ddf1* phenotype was caused by overexpression of a gene encoding a putative AP2 transcription factor, *DDF1* through repression of GA biosynthesis. The salt-stress responsiveness of *DDF1* and increased salt-stress tolerance of *DDF1*-overexpressing transgenic plants indicate the involvement of *DDF1* in stress tolerance.

Results

The ddf1 mutant is similar to GA-deficient mutants

Among the about 2500 individuals of the T₁ generation of our activation-tagged *Arabidopsis*, we found a dwarf plant with dark-green leaves. This mutant bolted later and the final height of the mature plant was smaller compared to wild-type *Arabidopsis* (ecotype Columbia; Col). In the T₂ generation, about three quarters of the mutant's progeny (178 of 234; Chi-square test: $P > 0.7$) showed the same traits (Figure 2a,b), indicating that this phenotype is conferred by a dominant mutation of a single gene. We named this mutant *ddf1*. The genomic Southern blot analysis uncovered that several copies of T-DNA were inserted into the genome (data not shown). Therefore we back-crossed the T₂ generation two times with Col. After self-fertilization of a dwarf BC2 plant with T-DNA in a single locus, an individual homozygous for the *ddf1* mutation was selected and its progeny was used for further studies.

The late-flowering phenotype of *ddf1* was observed regardless of day length, but it was enhanced under long day (LD) conditions compared to short day (SD) conditions (Figure 2c). Scanning electron microscopy showed that the epidermal cells of the stem were shorter in *ddf1* than in Col (Figure 2d). The epidermal cells of mature rosette leaves were smaller in *ddf1*, although small amounts of cells with normal size were found. These observations suggest that the dwarf phenotype of *ddf1* is mainly because of reduced cell size. Other characteristics, including germination rate, flower morphology and fertility, appeared to be normal (data not shown). The dwarfed and late-flowering phenotype observed in *ddf1* was similar to that of GA-deficient (e.g. *ga1-ga5*) and GA-insensitive mutants (e.g. *gai*; Koornneef and van der Veen, 1980; Koornneef *et al.*, 1985).

The phenotypes of ddf1 are rescued by exogenous GA₃

To examine the involvement of GA in the *ddf1* mutation, we investigated the responsiveness of *ddf1* to exogenous GA. Seeds of *ddf1* were germinated and grown on 1/2 MS plates containing 10⁻⁷, 10⁻⁶, or 10⁻⁵ M GA₃. The normal traits were restored partially in *ddf1* by 10⁻⁷ M GA₃ (data not shown) and fully by GA₃ at concentrations higher than 10⁻⁶ M (Figure 3a). Dwarfism of *ddf1* growing on soil was avoided by weekly spraying with 10⁻⁴ M GA₃; the final height of the restored *ddf1* was almost the same as that of Col (Figure 3b). The late-flowering phenotype was largely repressed by application of GA₃ (Figure 2c).

As well as GAs, BRs promote cell elongation, and the known BR-deficient or BR-insensitive mutants are dwarfs. To check the responsiveness of *ddf1* to BRs, we applied BR to *ddf1* by adding brassinolide to 1/2 MS plates. Growth of *ddf1* was partly restored by 10⁻¹⁰ M brassinolide, but full

recovery could not be observed at any concentration tested (up to 10^{-6} M; data not shown). De-etiolation is another characteristic of BR mutants (reviewed by Bishop and Koncz, 2002; Kauschmann *et al.*, 1996). *ddf1* seedlings grown in the dark etiolated like wild-type seedlings (data not shown). Thus, the *ddf1* phenotype is probably caused by deficiency of GA, not of BR.

Levels of bioactive GAs are decreased in *ddf1*

We measured the contents of endogenous GAs in matured plants of *ddf1* and Col using gas chromatography-selected

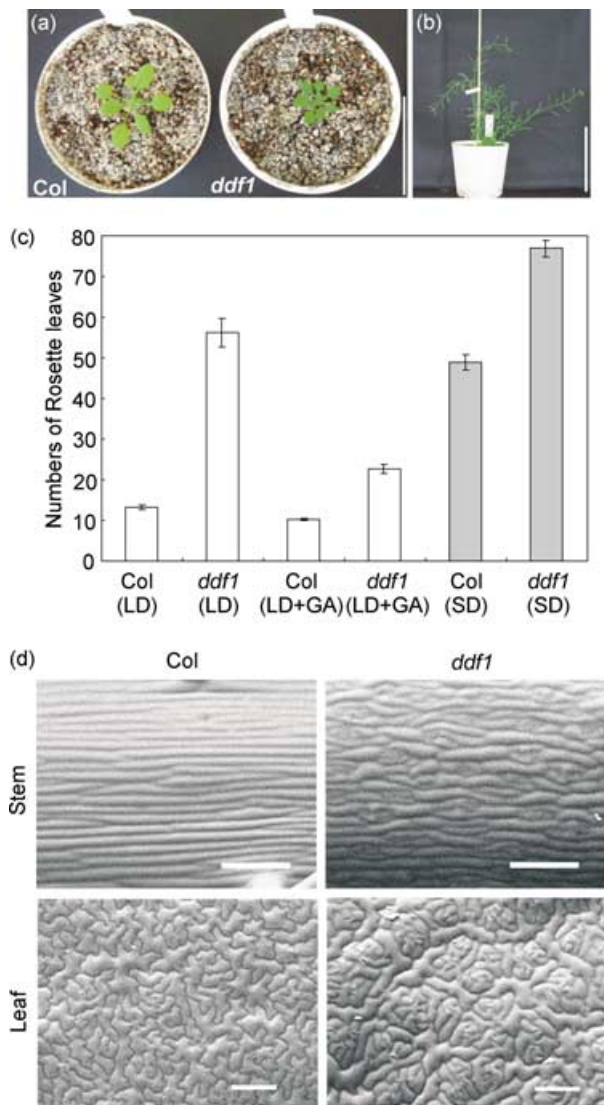


Figure 2. Phenotypes of the *ddf1* mutant. (a) Three-week-old seedlings of wild-type (Col) and *ddf1*. Bar = 5 cm. (b) A 10-week-old *ddf1* plant. Bar = 10 cm. (c) Numbers of rosette leaves of Col and *ddf1* at flowering stage. Plants were grown under LD or SD condition with or without spraying with 10^{-4} M GA₃. Error bars show SEs ($n = 16$ –38). (d) Scanning electron microscopic observation of the epidermal cells of the stem and leaf in Col and *ddf1*. Bar = 100 μ m.

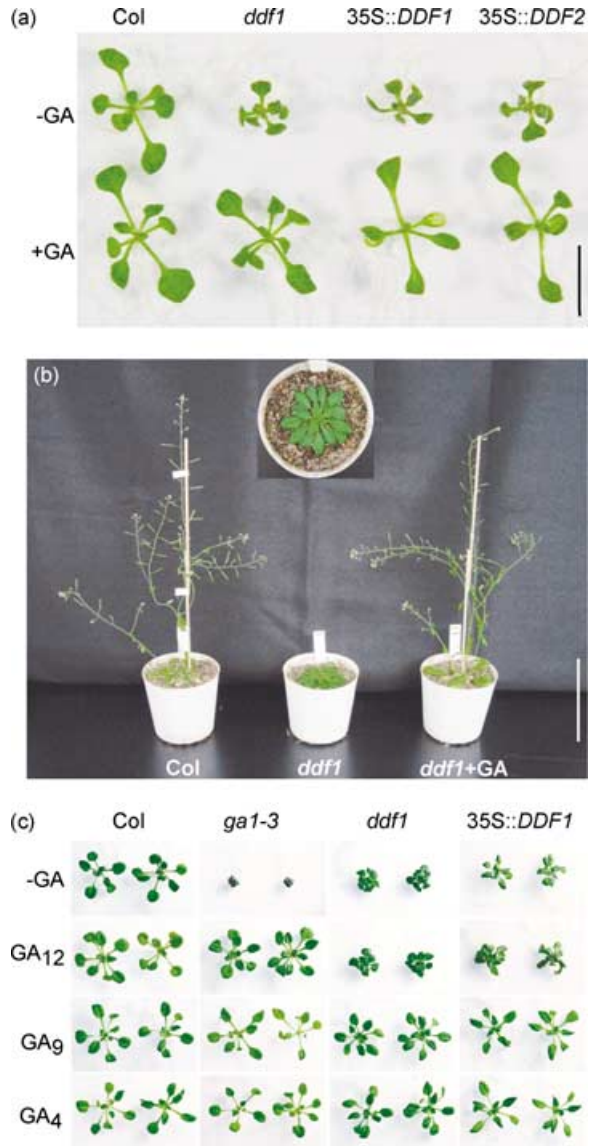


Figure 3. Restoration of wild-type traits in *ddf1* by exogenous GAs. (a) Fourteen-day-old seedlings of *ddf1* and transgenic plants overexpressing *DDF1* or *DDF2*. Each seedling was grown on 1/2 MS medium without (upper row) or with 10^{-6} M GA₃ (lower row). (b) Six-week-old plants of Col (left), *ddf1* (middle), and *ddf1* sprayed with 10^{-4} M GA₃ once every week (right). Bar = 10 cm. (c) Twenty-day-old seedlings of Col, *ga1-3*, *ddf1*, and 35S::*DDF1* treated with GA₄, GA₉, or GA₁₂. Each seedling was grown on the plate without (upper row) or with (lower rows) 2×10^{-7} M of each GA derivative.

ion monitoring (GC-SIM). As shown in Table 1, the contents of the bioactive GAs, GA₄ and GA₁, were only 11 and 17%, respectively, of those detected in Col, demonstrating that *ddf1* is a GA-deficient mutant. Interestingly, the contents of intermediates (GA₁₅, GA₄₄, GA₂₄, and GA₁₉) and end products (GA₉ and GA₂₀) of GA₂₀ox were significantly decreased in *ddf1*. In contrast, the levels of the substrates of GA₂₀ox, GA₁₂ and GA₅₃, were 182 and 109% of those

Table 1 Levels of GAs in Col and *ddf1* mutant (ng g⁻¹ FW)

	GA ₁₂	GA ₁₅	GA ₂₄	GA ₉	GA ₄	GA ₃₄	GA ₅₁	GA ₅₃	GA ₄₄	GA ₁₉	GA ₂₀	GA ₁	GA ₈	GA ₂₉
Col	2.22	0.54	6.15	0.15	0.37	0.77	0.30	0.35	0.25	1.01	0.04	0.06	0.26	0.07
<i>ddf1</i>	4.03	0.12	3.71	0.03	0.04	0.23	0.32	0.38	0.12	0.62	0.01	0.01	0.11	0.03
Ratio (<i>ddf1</i> /Col)	1.82	0.22	0.60	0.20	0.11	0.30	1.07	1.09	0.48	0.61	0.25	0.17	0.42	0.43

found in Col. The contents of deactivated catabolites of GA2ox (GA₅₁, GA₂₉, GA₃₄, and GA₈) were decreased or almost the same as in Col. A repeated experiment showed the similar tendency. These results indicate that the deficiency of bioactive GAs in *ddf1* is because of the inhibition of stepwise oxidation catalyzed by GA20ox, but not to the deactivation of bioactive GAs. Consistent results were obtained from the application of GA intermediates (Figure 3c). When growing on plates with 2×10^{-7} M GA₁₂, the dwarf phenotype of *ddf1* and 35S::*DDF1* were not affected. In contrast, 2×10^{-7} M GA₉ recovered dwarfism of *ddf1* and 35S::*DDF1* as well as GA₄. At the same concentrations all GA intermediates tested rescued dwarfism of *ga1-3*, which is defective in the first step of GA biosynthesis.

The ddf1 phenotype is conferred by overexpression of a putative AP2 transcription factor

As the *ddf1* mutation was dominant, the mutation was probably caused by the 35S enhancer in the T-DNA inserted. To test this possibility, we cloned genomic DNA fragments flanking the T-DNA insertion by plasmid rescue and performed genomic PCR. As expected, a strong linkage between the *ddf1* phenotype and the T-DNA insertion was revealed (data not shown). Southern blot analysis showed that in a single locus, at least two copies of T-DNAs were inserted in an inverted repeat orientation, as illustrated schematically in Figure 4(a). Therefore, the three deduced genes around the T-DNA insertion site appeared as prime candidates for the causal gene of the *ddf1* mutation. We constructed three kinds of transgenic plants, each carrying one of the genes under the control of the CaMV 35S promoter. Only transgenic plants carrying a putative AP2 transcription factor (AGI# At1g12610) exhibited the dwarfish and late-flowering phenotype typical of *ddf1* (Figure 3a), although the degree of growth reduction varied among lines. Dwarfism was prevented by exogenous GA₃. Furthermore, real-time RT-PCR showed about 15- and 140-fold higher accumulation of *DDF1* mRNA in seedlings of *ddf1* and 35S::*DDF1* line #4, respectively, than in the wild type (data not shown). Transgenic plants containing a 6.6-kbp *HindIII* fragment of *ddf1* genomic DNA, including the 35S-enhancer of the inserted T-DNA as well as the promoter and coding region of the putative AP2 transcription factor gene (Figure 4a), developed a similar phenotype (data not shown). We concluded that the *ddf1* phenotype

was conferred by overexpression of this putative AP2 transcription factor gene designated *DDF1*.

The predicted product of *DDF1* consists of 209 amino acids. It has an AP2 transcription factor domain and a putative nuclear localization signal in the N-terminal half, and also possesses a serine-rich domain in the C-terminal half (Figure 4b). We found a *DDF1* homolog (AGI# At1g63030) with 69% of identity at amino acid level in the *Arabidopsis* genome database, which we designated *DDF2* (Figure 4b). The predicted product of *DDF2* is 28 amino acids shorter than that of *DDF1* because of three gaps in the C-terminal portion. Interestingly, the location of the largest gap (24 amino acids) corresponds to the serine-rich region of *DDF1*. To determine whether *DDF2* is functionally analogous to *DDF1*, we generated transgenic plants expressing *DDF2* controlled by the 35S promoter. Phenotypically, 35S::*DDF2* plants were indistinguishable from *ddf1* or 35S::*DDF1* plants (Figure 3a). Furthermore, the development of the dwarf phenotype of 35S::*DDF2* plants was prevented by exogenous application of GA₃ just as it was in *ddf1* and 35S::*DDF1* plants. In the 35S::*DDF2* line #4,

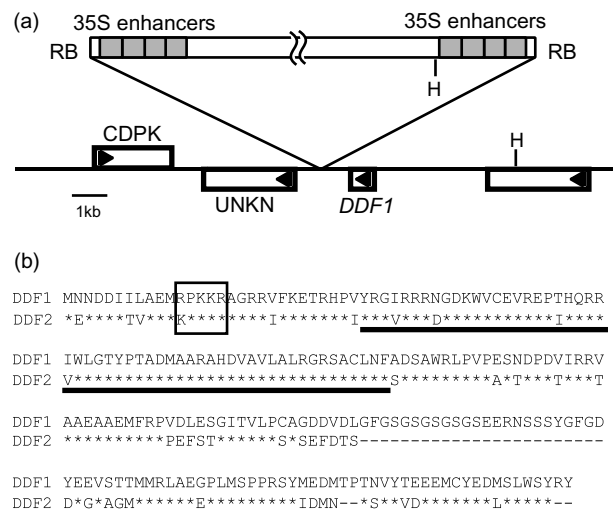


Figure 4. Arrangement of T-DNAs in the *ddf1* genome and sequence comparison of DDFs.

(a) Inserted T-DNAs and putative genes around the insertion site. H: *HindIII* site. Three kinds of transgenic plants, each overexpressing one of the genes shown as CDPK, UNKN, and *DDF1* were constructed (see the text). (b) Comparison of deduced amino acid sequences of *DDF1* and *DDF2*. Conserved AP2 domains are underlined. Box highlights a putative nuclear localization signal. Asterisks mark identical amino acids.

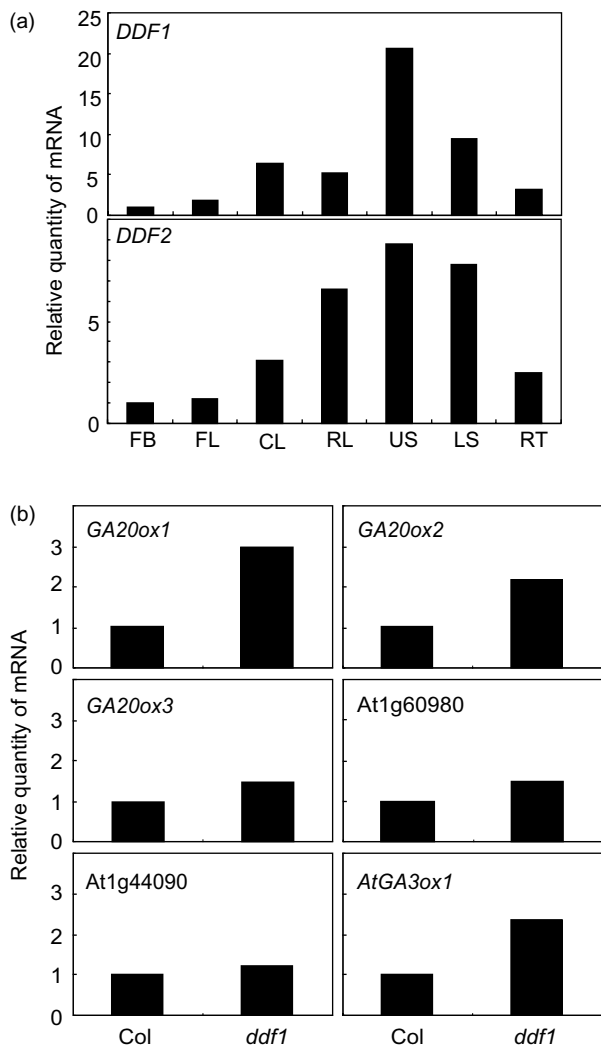


Figure 5. Comparison of the amount of transcripts by real-time RT-PCR. (a) Relative amount of transcripts for *DDF1* and *DDF2* in various tissues of mature wild-type plants. Flower buds (FB), flowers (FL), cauline leaves (CL), rosette leaves (RL), upper stem (US), lower stem (LS), root (RT). (b) Transcript levels of (putative) *AtGA20ox* and *AtGA3ox* genes in 11-day-old seedlings. Transcript levels are expressed relative to the level of transcripts in Col, which are assumed to be one.

DDF2 mRNA was accumulated up to about 180-fold as compared to the wild type (data not shown).

Expression analysis of *DDF1* and *DDF2*

The tissue specificities of the expression of *DDF1* and *DDF2* were similar in mature plants. Highest expression levels were found in stem. Considerable levels were detected in cauline leaves and rosette leaves. In flowers and flower buds, the transcripts were relatively rare (Figure 5a).

It is known that biosynthesis of GA is negatively regulated by bioactive GA. We measured the relative amounts of *DDF* transcripts in wild-type plants treated with 10^{-4} M GA_3 for

various times (1–24 h). However, significant induction of *DDFs* was not observed at any time (data not shown). Therefore, *DDFs* are unlikely to participate in the negative feedback regulation of GA biosynthesis.

The ddf1 mutation does not decrease the expression of *GA20ox* genes

To date, three *GA20ox* genes are known in *Arabidopsis*: *AtGA20ox1* (*GA5*), *AtGA20ox2*, and *AtGA20ox3* (Phillips *et al.*, 1995). Two additional genes, *AG1# At1g60980* and *At1g44090*, are considered putative *GA20ox* genes because of their high sequence homology with *AtGA20ox1* (47 and 29%, respectively, at amino acid level). As *DDFs* are putative transcription factors, an obvious possibility is that the inhibition of the biosynthetic steps catalyzed by *GA20ox* in *ddf1* is caused by downregulation of *GA20ox* genes. To test this hypothesis, we estimated the expression levels of *GA20ox* genes in seedlings using real-time RT-PCR. No decrease was detected in expression levels of all these genes in *ddf1* as compared to the wild type. On the contrary, the transcripts of *AtGA20ox1*, *AtGA20ox2*, and *AtGA20ox3* increased in *ddf1* (Figure 5b). *AtGA3ox1* was also upregulated. These findings were confirmed in *35S::DDF1* and *35S::DDF2* plants (data not shown). Thus, the inhibition of GA biosynthesis in *ddf1* is not because of a transcriptional repression of *GA20ox* genes. Upregulation of the genes for *GA20ox* and *GA3ox* have been reported previously in GA-deficient and GA-insensitive mutants (Cowling *et al.*, 1998; Xu *et al.*, 1995) and are commonly thought to indicate positive feedback mechanisms.

DDFs are closely related to *DREB1/CBF* gene family

To characterize the relation between *DDFs* and other AP2 transcription factors in *Arabidopsis*, we constructed a phylogenetic tree from amino acid sequence data of the conserved AP2 domains (Figure 6a). *DDF1* and *DDF2* were positioned on a branch close to the *DREB1/CBF* family, a group of transcription factors responsive to cold or dehydration stress (Gilmour *et al.*, 1998; Haake *et al.*, 2002; Liu *et al.*, 1998; Stockinger *et al.*, 1997). High degrees of sequence homology are spread throughout various sequence regions among *DREB1/CBF* genes, but they seem restricted to the AP2 domain if *DDF* and the *DREB1/CBF* family are compared. Of noteworthy, transgenic plants overexpressing *DREB1/CBFs* showed not only increased tolerance for abiotic stresses but also dwarf phenotypes (Gilmour *et al.*, 2000; Haake *et al.*, 2002; Kasuga *et al.*, 1999; Liu *et al.*, 1998), which resembled the traits of *ddf1*. The *tiny* mutant is a semidominant dwarf mutant caused by overexpression of another AP2 transcription factor, *TINY*. It resembles *ddf1*, but the sequences of *DDFs* seem distantly related to that of *TINY*. Unlike *ddf1*, exogenous GA could

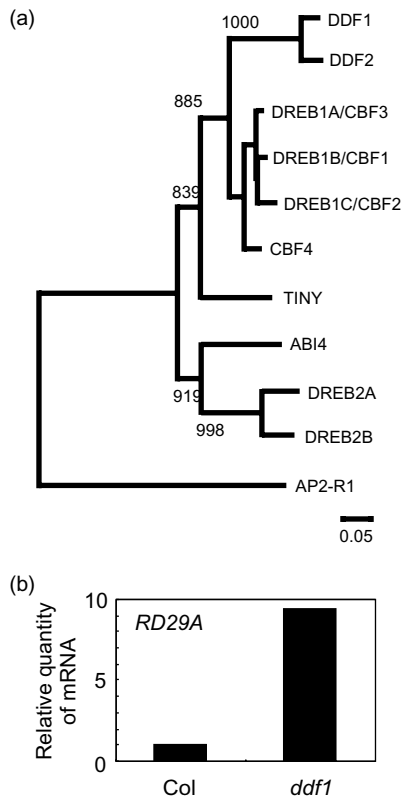


Figure 6. Phylogenetic tree of DDF-related proteins and expression of *RD29A* in *ddf1*.

(a) Phylogenetic tree of DDFs and related AP2 transcription factors found in the *Arabidopsis* genome. The tree was constructed from amino acid sequences of the conserved AP2 domain by the neighbor-joining method. The bootstrap values (>800) are shown at the nodes. AP2-R1 (Jofuku *et al.*, 1994) was added as an out-group.

(b) Transcript levels of *RD29A* in 11-day-old seedlings. The transcript level in Col is arbitrarily set as one.

not rescue the development of a dwarf phenotype in *tiny* (Wilson *et al.*, 1996).

DREB1/CBFs induce cold-responsive (COR) genes that have C-repeat/dehydration-responsive element (CRT/DRE) core motifs (A/GCCGAC) in their promoters (Sakuma *et al.*, 2002). To test whether *DDF1* could upregulate these genes, we analyzed the expression of *RD29A/COR78*, one of the *DREB1/CBF*-inducible COR genes (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Yamaguchi-Shinozaki and Shinozaki, 1994). The transcripts of *RD29A/COR78* were about ninefold more abundant in *ddf1* seedlings than in the wild type (Figure 6b). In *ddf1* plants, the transcript level of *DREB1A/CBF3*, a major member of the *DREB1/CBF* family, was not significantly changed (data not shown).

DDF1 responds strongly to high-salinity stress

Recently, Sakuma *et al.* (2002) reported that expression of *DDF1* (designated as *DREB1F*) in *Arabidopsis* roots is

induced when treated with 250 mM sodium chloride. We monitored the time course of the accumulation of *DDF1* mRNA under high salinity condition by real-time RT-PCR. When *Arabidopsis* seedlings were transferred from plates onto filter papers soaked with water, the level of *DDF1* mRNA in roots was upregulated transiently at 30 min and returned to basal level at 1 h (Figure 7a). When transferred onto filter papers soaked with 250 mM sodium chloride, the level of *DDF1* mRNA significantly exceeded that of water control at 1 h and continuously increased for at least 5 h to 956-fold. The significant accumulation of *DDF2*

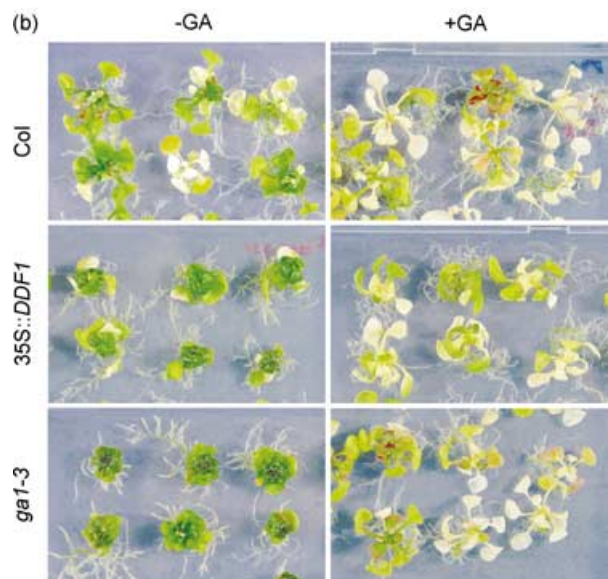
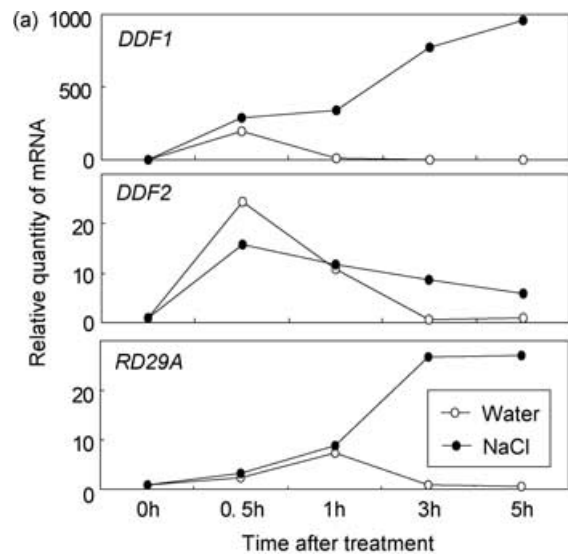


Figure 7. Effects of high-salinity stress.

(a) Transcript levels of *DDFs* and *RD29A* in Col treated with water or 250 mM sodium chloride.

(b) Physiological changes of plants transferred to plates containing 170 mM sodium chloride. Photographs were taken on day 13 post-transfer. +GA indicates the treatment with 10^{-6} M GA_3 .

Table 2 Survival rates of plants grown under high-salinity condition

	Total plants	Survival	Rate (%)
Col	55	29	52.7
Col (+GA)	60	17	28.3 ^a
35S::DDF1	60	57	95.0 ^a
35S::DDF1 (+GA)	60	24	40.0
<i>ga1-3</i>	55	51	92.7 ^a
<i>ga1-3</i> (+GA)	55	21	38.2

Two-week-old plants grown on 1/2 MS plates were transferred to plates containing 170 mM sodium chloride. Survived plants with green apical buds were checked on day 13 post-transferring. +GA indicates plants grown with 10^{-6} M GA₃ throughout their lives.

^aStatistical significance ($P < 0.001$) compared with Col by Chi-square test.

mRNA was also observed at 3 and 5 h. However, *DDF2* was less responsive to salt stress compared to *DDF1*. The stress-related gene *RD29A/COR78* was significantly induced at 3 h by this treatment.

Transgenic plants overexpressing DDF1 show increased tolerance to high-salinity stress

To clarify the physiological roles of *DDF1* under high-salinity condition, 35S::*DDF1* transgenic plants grown on 1/2 MS plates for 2 weeks were transferred to 1/2 MS plates containing 170 mM sodium chloride. Figure 7(b) shows the states of plants on day 13 after transfer. Among wild-type plants, we often found damaged plants with white apical buds that were doomed to be dead. On the other hand, most of 35S::*DDF1* had green apical buds and survived. The survival rate of 35S::*DDF1* was much higher (95.0%) than that of Col (52.7%; Table 2), suggesting that *DDF1* relates to stress tolerance. Interestingly, the tolerance to high-salinity stress was apparently reduced when 10^{-6} M GA₃ was exogenously applied (28.3% in Col and 40.0% in 35S::*DDF1*). The involvement of GA to the stress tolerance was confirmed using the GA biosynthetic mutant *ga1-3*. This mutant showed higher tolerance to high-salinity stress (92.7%) than Col did, and the tolerance was decreased by exogenous GA₃ (38.2%).

Discussion

In this study, we have characterized the dominant mutant *ddf1*, which seems to be a GA-deficient mutant for the following reasons. First, the phenotypic characteristics of *ddf1* include traits typical to known GA-deficient mutants, namely shortened hypocotyls and petioles, small dark-green leaves, and late-flowering. Second, the development of the dwarf phenotype of *ddf1* was fully prevented by exogenous GA₃. Moreover, flowering occurred earlier in hormone-treated *ddf1* plants (Figures 3b and 2c). Third,

quantitative analysis of endogenous GAs proved that the levels of the bioactive compounds GA₄ and GA₁ were substantially reduced in *ddf1* (11 and 17%, respectively; Table 1). It should be noted that *ddf1* germinated and flowered under SD conditions and retained fertility. In this respect, *ddf1* resembles semidwarf mutants such as *ga4* and *ga5*, which have 10–30% of bioactive GAs compared to the wild type (Talon *et al.*, 1990), and differs from the severe dwarf mutants, *ga1-3*, which cannot germinate and bolt under SD conditions without exogenous application of GA (Koornneef and Van der Veen, 1980; Wilson *et al.*, 1992). These facts suggest that GA biosynthesis is partially inhibited in *ddf1*.

Quantification of endogenous GA derivatives revealed that in *ddf1*, the intermediates and final products of GA20ox were decreased, whereas the substrates of GA20ox were increased or not significantly affected (Table 1). The oxidation steps catalyzed by GA20ox are known to be regulated by the transcriptional repressor NTH15 in tobacco (Sakamoto *et al.*, 2001). Therefore, we speculated that GA20ox genes were downregulated in *ddf1*, as it is the case in transgenic tobacco carrying 35S::*NTH15* (Tanaka-Ueguchi *et al.*, 1998). However, none of the three known GA20ox genes (*AtGA20ox1*, *AtGA20ox2*, and *AtGA20ox3*) and two putative ones was repressed in *ddf1* (Figure 6c) and in 35S::*DDF1* plants (data not shown). Rather, some of the genes were enhanced, as also observed in other GA-deficient mutants or in plants treated with inhibitors of GA biosynthesis (Cowling *et al.*, 1998; Xu *et al.*, 1995). Therefore, an involvement of *DDFs* in the transcriptional repression of GA20ox genes seems unlikely. The inhibition of GA biosynthesis might be caused by other mechanisms like post-transcriptional modification of GA20ox, induction of an inhibitory subunit of GA20ox, or production of unknown enzymes that catabolize C₁₉-GA intermediates (GA₁₅, GA₄₄, GA₂₄, GA₁₉, GA₉, and GA₂₀).

ddf1 mutants seem to suffer from increased or ectopic expression of the gene for the putative transcription factor *DDF1*, as transcripts of *DDF1* accumulated in *ddf1* and 35S::*DDF1* transgenics mimicked *ddf1*. The homologous gene *DDF2* induced identical symptoms when expressed under the 35S promoter. On the phylogenetic tree, we constructed, *DDFs* were located close to DREB1/CBF transcription factors (Figure 6a). Regulation of transcription by *DDFs* has not been directly proven yet. However, *RD29A/COR78*, a gene controlled by DREB1 transcription factors, is upregulated in *ddf1* seedlings without corresponding up-regulation of *DREB1A/CBF3* (Figure 6b). Accumulation of transcripts of *KIN1* and *COR47*, other DREB1-regulated genes, was also detected by expression profiling using the Affimetrix Genechip 8K (unpublished data). These suggest that *DDF1* might recognize the CRT/DRE-like element as DREB1/CBF transcription factors do and have transcriptional activation activity.

DREB1/CBFs are known to have important roles for cold acclimation or dehydration tolerance through transcriptional activation of stress-related genes (Haake *et al.*, 2002; Shinozaki and Yamaguchi-Shinozaki, 2000). The question arises whether DDFs function in stress tolerance. *DDF1* was strongly induced under high-salinity condition (Sakuma *et al.*, 2002; Figure 7a). Stress-related genes, including *RD29A/COR78*, were highly expressed in *ddf1*. Moreover, transgenic plants overexpressing *DDF1* showed increased tolerance to high-salinity stress (Figure 7b; Table 2). These suggest that DDF1 relates to salt-stress response through regulating stress-related genes.

Exogenous application of 10^{-6} M GA₃ recovered dwarfism of 35S::*DDF1* (Figure 3a,b). Of noteworthy, when grown with 10^{-6} M GA₃, Col and 35S::*DDF1* decreased their tolerance to high-salinity stress (Figure 7b; Table 2). A GA biosynthetic mutant, *ga1-3*, showed improved tolerance to high-salinity stress as well as 35S::*DDF1* and exogenous GA₃ decreased the tolerance. These indicate that the levels of active GAs affect the viability under stress condition. It should be noted that plantlets of 35S::*DDF1* and *ga1-3* were morphologically different compared to Col. The increased tolerance may be caused by low transpiration rates and/or reduced uptake of salt because of smaller surface areas. Other changes, including changes of physiological states, induced by decreased GA may have influence on the stress tolerance. Vettakkorumakankav *et al.* (1999) also reported the possible relation between endogenous GA contents and stress tolerance in barley.

According to our findings, it is conceivable that in response to high-salinity stress, *Arabidopsis* represses GA biosynthesis through the induction of *DDF1* and positively changes its morphological and/or physiological states for stress protection. However, it is still unclear whether the plants actually modulate their GA contents in response to environmental stress. To clarify the question, it is necessary to measure the levels of endogenous active GAs in the plants exposed to stress.

Intriguingly, dwarfism is observed in transgenic *Arabidopsis* overexpressing *DREB1A/CBF3*, *DREB1B/CBF1*, or *CBF4* (Gilmour *et al.*, 2000; Haake *et al.*, 2002; Kasuga *et al.*, 1999; Liu *et al.*, 1998). The development of dwarf phenotypes was also found in transgenic tomato overexpressing *Arabidopsis DREB1B/CBF1*, and it was prevented by exogenous application of GA₃ (Hsieh *et al.*, 2002). These suggest that an inhibition of GA biosynthesis is a function common to *DREB1/CBF* genes. However, DNA microarray analysis using the Affimetrix Genechip 8K did not detect the changes in transcript levels of GA-related genes in transgenic *Arabidopsis* overexpressing *DREB1A/CBF3*, *DREB1B/CBF1*, or *DREB1C/CBF2* (Fowler and Thomashow, 2002), although it detected the induction of GA-related genes, including *AtGA20ox1*, *AtGA20ox2*, and *AtGA3ox1* in *ddf1* (unpublished data; also shown by real-time RT-PCR in Figure 5b). Measurements of

GA contents and detailed expression analysis of GA-related genes in the transformants overexpressing *DREB/CBFs* will help to resolve this discrepancy.

Experimental procedures

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col was used as the wild type. Plants were grown on soil or 1/2 MS medium plates (Murashige and Skoog, 1962) under LD (16 h light/8 h dark) or SD (8 h light/16 h dark) conditions at 22°C. *ddf1* was found in one individual among about 2500 plants of the T₁ generation of *Arabidopsis* transformed with the activation-tagging vector pPCVICEn4HPT (Hayashi *et al.*, 1992). The *ga1-3* mutant was originally found in Landsberg *erecta* background (Koorneef and van der Veen, 1980). The *ga1-3* allele backcrossed to Col-0 six times (courtesy of Dr Tai-ping Sun, Duke University, USA) was used in the experiment.

Scanning electron microscopy

Rosette leaves and stems were harvested from mature plants and observed directly by a scanning electron microscope JSM-5800 (JEOL Inc. Tokyo, Japan) equipped with a cooling stage.

Hormonal treatment

For application of GA, plants were grown on 1/2 MS plates containing 10^{-7} , 10^{-6} , or 10^{-5} M GA₃. For application of BR, plants were grown on plates with 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , or 10^{-6} M brassinolide. Growth of plants was monitored for 14 days post-seeding. For application of GA to plants growing on soil, 10^{-4} M GA₃ was sprayed once a week starting on the 10th day post-seeding. For application of GA intermediates, seeds were stratified in the presence of 5×10^{-5} M GA₄ in the dark at 4°C for 4 days. The seeds were washed with sterile water five times to get rid of excess GA₄, placed on MS agar media with or without 2×10^{-7} M GA₄, GA₉, or GA₁₂, and then incubated under continuous white light at 22°C for 20 days before the pictures were taken.

Quantification of GA derivatives

Aerial parts of Col and *ddf1* plants grown on soil under LD conditions were harvested just before flowering and were stored in liquid nitrogen at -70°C. Extraction and analysis of GAs were performed as described previously (Gawronska *et al.*, 1995; Silverstone *et al.*, 2001). Briefly, ²H-labeled GAs were added to each sample before extraction as internal standards. After purification, the samples were analyzed by GC-SIM on a mass spectrometer (Auto Mass, JEOL Inc.).

Plasmid rescue

For *DDF1* cloning, 2 µg of genomic DNA isolated from 10-day-old *ddf1* plants was digested with *EcoRI* or *Clal*, respectively. The DNA was purified by phenol and chloroform extraction, concentrated by ethanol precipitation, and self-ligated with T4-DNA ligase (Takara, Otsu, Japan) at 16°C overnight. The ligated DNA was precipitated with ethanol, dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer, and one-tenth of it was electroporated into DH10B

competent cells (Invitrogen Corp., Carlsbad, CA, USA). Colony selection was carried out on Luria-Bertani (LB) plates containing Ampicillin. Genomic DNA fragments obtained were subcloned into conventional vector plasmids and sequenced.

Transgenic plant construction

To construct transgenic plants overexpressing *DDFs*, coding regions of *DDF1* and *DDF2* were amplified from genomic wild-type DNA using a set of 5'-CCTCTAGAATGAATAATGATGATAT-TATTC-3' (*Xba*I site was underlined) and 5'-CCCTGCAGTTAA-TATCTGTAACCTCC-3' (*Pst*I site underlined) primers, and a set of 5'-CCTCTAGAATGGAAAACGACGATAT-3' (*Xba*I site underlined) and 5'-CCCTGCAGTTAGTAACTCCAAAGTG-3' (*Pst*I site underlined) primers, respectively. The PCR products were ligated into T-vector plasmids. After confirmation of the nucleotide sequence, the plasmid DNAs were digested with *Xba*I and *Pst*I, and DNA fragments containing *DDFs* were inserted between the CaMV 35S promoter and the nopaline synthase (NOS) terminator of the pCGN binary vector. *Agrobacterium* was transformed with these constructed binary plasmids, and the T-DNA regions were transferred into *Arabidopsis* by vacuum infiltration (Bechtold *et al.*, 1993).

Estimation of mRNA by real-time RT-PCR

Total RNA of *Arabidopsis* was isolated using the RNeasy extraction kit (Qiagen, Valencia, CA, USA), including a DNA elimination step. Two micrograms of total RNA were used for cDNA synthesis using the Prostar™ first strand RT-PCR kit (Stratagene, La Jolla, CA, USA) with a random hexamer primer. Two microliters of a fivefold dilution of the reaction was used subsequently for PCR. Real-time RT-PCR was performed with the ABI PRISM 7700 sequence detection system with TaqMan PCR master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All data were normalized with respect to 18S rRNA, which was measured as a control in every experiment. Specific primers and probes were designed as follows: *DDF1* (forward: 5'-GATCCGCATGTTT-GAATTCG-3', reverse: 5'-ATCATTGGATTCCGGCACC-3', probe: 5'-CGACTCCGCTTGCGCGCTTC-3'), *DDF2* (forward: 5'-CCCTCAGC-CAGTGAGTTTGAC-3', reverse: 5'-TCCGCGAGCCTCATCATC-3', probe: 5'-CGTCCGGATGAAGGAGTCGCTGGAA-3'), *GA20ox1* (forward: 5'-CCGCTCAAATCCGTTCAAG-3', reverse: 5'-CCTTCCCAA-ATGGCTGAAAC-3', probe: 5'-TTACTTCTGCGATGCGTTGGGACA-TG-3'), *GA20ox2* (forward: 5'-TGCTCACCGTTTGTATGGAAG-3', reverse: 5'-CACCGGGTTTTCTCTGAGCTT-3', probe: 5'-ACATGCCT-CTCGCCGGCAAACAG-3'), *GA20ox3* (forward: 5'-CTCCAAGTCC-CACTCATAGACCTAG-3', reverse: 5'-TAGCCTCCGATGCCAAGC-3', probe: 5'-CGGTTTCTCTCCGGCGACTCG-3'), *At1g60980* (forward: 5'-CAATCCTCCAAGTCCCTGTCA-3', reverse: 5'-GCCTCCGAGAC-CAATAATGG-3', probe: 5'-CCTCGCAGGCTTCTCTCCAACG-3'), *At1g44090* (forward: 5'-GGGCTTAGAGGTCTTTGCCG-3', reverse: 5'-ACGACAAGAGCACCAGGACG-3', probe: 5'-AGGTAGTTGGCA-GACCGTTGCCCC-3'), *DREB1A* (forward: 5'-GCTGACTCGGCTTG-GAGACT-3', reverse: 5'-CCGCCTTTGGATGTCCTT-3', probe: 5'-CGAATCCCGGAATCAACTTGCCG-3'), and 18S rRNA for normalization (forward: 5'-AGTCATCAGCTCGCTTGAC-3', reverse: 5'-TCAATCGGTAGGAGCGACG-3', probe: 5'-TCCCTGCCCTTTGTACA-CACCGC-3'). All probes were labeled with FAM and TAMRA at the 5' and 3' ends, respectively. Deviating from the procedure described, *GA3ox1* (forward: 5'-CGGTCCGAGACAGGTGTGT-3', reverse: 5'-CCTTCGACCACATTTGCTT-3') and *RD29A/COR78* genes (forward: 5'-TCGACAAGGATGTGCCGAC-3', reverse: 5'-CTGATGCCTCACCG-TATCCA-3') were quantified using the SYBR Green PCR Master Mix (Applied Biosystems).

Phylogenetic analysis

Constructions of alignment of amino acid sequences and neighbor-joining tree with bootstrap re-sampling were conducted by the CLUSTALW program (Thompson *et al.*, 1994).

Treatment with high-salinity stress

Two-week-old seedlings grown on 1/2 MS plates under constant light were transferred onto filter papers soaked with 250 mM sodium chloride or water and incubated at 22°C. 0.5–5 h later, roots were harvested into liquid nitrogen to estimate mRNA.

In the stress tolerance assay, 2-week-old seedlings grown on 1/2 MS plates with or without 10^{-6} M GA_3 were transferred to plates containing 170 mM of sodium chloride with or without 10^{-6} M GA_3 . After incubation at 22°C for 13 days under constant light, effects of high-salinity stress were evaluated.

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