Integrative Study on Proteomics, Molecular Physiology, and Genetics Reveals an Accumulation of Cyclophilin-Like Protein, TaCYP20-2, Leading to an Increase of Rht Protein and Dwarf in a Novel GA-Insensitive Mutant (gaid) in Wheat

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Dwarfism with a “Green Revolution” phenotype is a desirable agronomic trait for crop cultivators as associated with increased yield, improved lodging resistance and higher fertility. Few dwarf mutants of hexaploid wheat (Triticum aestivum), except for Rht-B1 and Rht-D1, have been identified. Here, we report on a novel dwarf natural wheat mutant, which is identified as a gibberellic acid (GA)-insensitive dwarf (gaid) mutant for its semidominant blocking GA signaling pathway. Physiological and morphological investigations showed that the shoot elongation of gaid mutant plants is insensitive to exogenous GA3 treatment. Expression of TaGA20ox1 in the gaid mutant was enhanced after GA3 treatment. The short stem of gaid resulted from reduced cell elongation. The transcript expression of Rht, encoding a DELLA protein negatively regulating GA signaling in wheat, displayed similar patterns between gaid and wild type. Contrarily, the degradation of Rht induced by GA3 treatment was suppressed in the mutant. 2-DE screening assay showed that the expression patterns of the mutant, as well as their responses to GA3, were changed as compared with the wild type. In the mutant, one of enriched proteins was identified as TaCYP20-2 by Q-TOF MS approach and immunoblotting. TaCYP20-2 was localized in the chloroplast and cell plasma membrane. The transcript of TaCYP20-2 was higher in gaid than that in wild type. Molecular genetic data showed that overexpression of TaCYP20-2 in wheat resulted in a dwarfism similar to that of gaid. It suggests that TaCYP20-2 is a new member that regulates wheat stem development mediated by DELLA protein degradation of GA signaling pathway.

Keywords: dwarf mutant gaid • gibberellin • proteomics • cyclophilin • DELLA protein • Triticum aestivum (wheat)

1. Introduction

Since the 1960s, agriculture has developed new varieties of grain crops with short stems and cultivation, the “Green Revolution”, to substantially increase crop yields.1 Dwarfism is a desirable characteristic for grain crops breeding. Dwarf varieties are more resistant to the damaging effects of wind and rain that cause stem lodging.2 Therefore, dwarf cereal is usually considered as a potential variety for increasing grain production with a less expense of biomass.

Dwarfism is commonly caused by mutations in genes regulating the biosynthesis or the signaling transduction of plant hormone gibberellic acid (GA). Various dwarf mutants in Arabidopsis (Arabidopsis thaliana; ga1-3 and gai), maize (Zea mays; d8), rice (Oryza sativa; sd1 and slr1), barley (Hordeum vulgare; sln1), and wheat (Triticum aestivum; Rht-B1 and Rht-D1) have been characterized and classified as being deficient or insensitive to the plant hormone GA3.3–9 Mutants severely defective in GA biosynthesis, such as the Arabidopsis mutant ga1-3, contain extremely low levels of bioactive GA and cannot germinate without GA treatment. The ga1-3 is a null gene encoding ent-copalyl diphosphate synthase in an early step of the GA biosynthesis pathway,9 so that the GA-deficient phenotypes of the ga1-3 mutant can be rescued by application of exogenous GA3. Besides, rice semidwarf sd1 mutant which contains a defective gibberellin 20-oxidase gene has a phenotype similar to ga1-3.8

GAI, D8, SLR1, SLN1, and Rht encode members of the DELLA subfamily within the GRAS family of plant regulatory proteins, which negatively regulate GA signaling transduction. Truncated...
Dwarf GA-Insensitive Mutant in Wheat

mutations of GAI/D8i/SLR1/Rht in the N terminal of DELLA proteins display a GA-insensitive response and gain the function to constitutively repress GA signaling.3,4,10,11 These dominant dwarf mutants fail to grow rapidly in response to GA treatment, but can be further dwarfed by GA biosynthesis inhibitor. In contrast to GA-insensitive phenotype in the gain-of-function mutants, loss-of-function mutants of barley shl1 and rice slr1 show a constitutive GA response phenotype with an elongated stem and leaf.6,12 Other dwarf GA-insensitive mutants are the repression of shoot growth (rsg) in tobacco (Nicotiana tabacum L.), photoperiod-responsive1 (phor1) in potato (Solanum tuberosum), dwarf1 (d1) and GA-insensitive dwarf1 (gid1) in rice.13–16 The proteins including RSG, PHOR1, D1, and GID1 function as positive regulators of GA signaling. GID1 is a soluble receptor mediating GA signaling in rice and shares sequence similarity with a hormone-sensitive lipase (HSL).13,17 The double mutant of gid1 and slr1 shows GID1 acting upstream of SLR1 in the GA signaling pathway, and SLR1 as a DELLA protein in rice was not degraded in the gid1 mutant on treatment with GA. The GA-insensitive dwarf mutants sleepy1 (syl1) in Arabidopsis and GA-insensitive dwarf2 (gid2) in rice revealed new insight into the GA signaling pathway. The SLY1 and GID2 proteins, which are homologous F-box proteins as a subunit of the SCF (Skp1/cullin/F-box) E3 ligase complex promote GA-dependent degradation of DELLA proteins in the ubiquitin proteasome pathway.18,19 Binding GA with GID1 induces the interaction between the DELLA proteins and GID1 and the degradation of DELLA proteins mediated by the F-box protein.17

Immunophilins consist of two subfamilies, cyclophilins and FK506-binding proteins (FKBPs). The members of the cyclophilin family appear in all kinds of species such as bacteria, fungi, insects, plants and animals, and are present in all subcellular compartments.20 The cyclophilin family modulates diverse processes, including protein folding, protein degradation, mRNA processing, apoptosis, development, stress responsiveness and receptor signaling. Moreover, the expression of cyclophilin is induced by both biotic and abiotic stresses.21 The plant cyclophilins were identified in the 1990s in diverse crops such as tomato (Lycopersicon esculentum), maize and oilseed rape (Vitis vinifera).22 They consist of a wide variety of single and multidomain isoforms varying by cellular locations and functions but mainly focus on participating in photosynthesis in chloroplasts and regulation of plant development.23 For example, TLP20, the spinach (Spinacia oleracea) cyclophilin protein, is the major peptidyl-prolyl cis–trans isomerase (PPIase) and protein-folding catalyst in the thylakoid lumen.23 The multidomain spinach cyclophilin TLP40 can be associated with PSII-specific protein phosphatase within the thylakoid membrane and regulate its activity, which is an essential process coinciding with phosphatase activation and dephosphorylation of PSII reaction center proteins.24,25 A few reports in the literature revealed the relationship between immunophilin-type PPIase and cell elongation. The Arabidopsis FKBP42 loss-of-function mutant twisted dwarf1 (twd1) displays a drastic reduction of cell elongation combined with a disoriented growth behavior of all plant organs.26 Lacking a cyclophilin, CYP40, causes a defect in the transition from the juvenile to adult stages of vegetative development and inflorescence morphology in Arabidopsis.27 Mutations in PAS1 (AtFKBP70) leading to tumor growth in plants indicates that FKBP70 is required for correct cell division and cell differentiation.28

CYP71 regulates plant morphogenesis and serves as a histone remodeling factor involved in chromatin-based silencing.29

Wheat is an important cereal crop with a complex hexaploid background (approximately 17 000 Mb), which causes difficulties in genomic mutation, gene cloning and genomic modification. The transcriptome analysis of gene expression at the mRNA level has contributed greatly to characterizing mutants of Arabidopsis and rice. In contrast, obstacles exist in studying the gene expression of wheat. Moreover, the level of mRNA does not always correlate well with the level of protein, the key player in the cell, as a result of post-transcriptional regulation mechanism. Therefore, proteome studies aiming at the complete set of genome-encoded proteins may complement the shortages of the transcriptome approaches. Here, we provide a successful explore using integrative studies of proteomics, physiology and molecular genetics to resolve the mutant with complex genetic background. We screened and characterized a new dwarf wheat mutant named as gaid (GA-insensitive dwarf) which was insensitive to GA. Proteomics approaches can provide new insights into the dwarfism of the gaid wheat mutant. Our comparative proteome analysis identified the accumulation of a cyclophilin protein, TaCYP20-2, in dwarf wheat. The transgenic wheat plants with TaCYP20-2 overexpression show a reduced height phenotype similar to the gaid mutant. The novel mechanism provides a new way to create a dwarf wheat cultivar for agricultural applications.

2. Materials and Methods

2.1. Isolation of gaid Wheat Mutant. A dominant dwarf wheat mutant was screened from the progenies of a transgenic wheat line (background Triticum aestivum cv. Jingdong 1) by the pollen tube transformation method.30,31 It was named as gaid, a GA-insensitive dwarf because of its physiological response. Experiments showed this mutation irrelevant to the foreign DNA sequence and relevant to natural mutagenesis. The gaid wheat seeds used in the experiments were the fifth generation of the origin mutant, which showed stable genetic characters. Local cultivars of winter wheat as hybrid parents, such as DN3214, ND99–5009, Jing411, and BAIU3338 were crossed with gaid.

2.2. Plant Materials and Growth Conditions. In field experiments, wild-type and gaid seeds were sown in the fall. In the following spring, the seedlings were sprayed with or without 100 µM GA3 (Sigma, St. Louis, MO, USA) for 4 weeks.

To investigate the role of GA3 and paclobutrazol (PAC) in leaf sheath elongation, seeds were surface-sterilized for 20 min with a 2% NaClO solution, washed with water and then placed on moistened papers for germination. After the seeds germinated for 2 days, seedlings with a uniform shoot length for the wild type and gaid mutants were selected (providing 30 seedlings per sample) and transferred to solution media, 1/2 Murashige and Skoog medium (pH 5.2), with the appropriate concentration of GA3 and PAC. The hydroponic medium was replaced with a new solution once every 3 days. Thirteen days later, the first leaf sheath length was determined in both the wild type and gaid.

2.3. Imaging of Leaf Sheath Cell Size. To examine cell arrangement and size, 5-mm-long fragments of the first leaf sheath apex of 7-day-old wheat seedlings were incubated in FAA buffer (10% glacial acetic acid, 10% 40% formalin, 80% 70% alcohol) for 48 h, then transferred into DL-lactic acid for 24 h. The length of epidermal cells in the leaf sheath was measured.
by use of a differential interference contrast (DIC) microscope (Zeiss) at 10 × magnification.32

2.4. Semi-quantitative PCR and Quantitative Real-time PCR.

Total RNA was extracted from shoots of 15-day-old of wild-type and gaid seedlings by use of Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was generated using the Reverse Transcriptase kit (TaKaRa, Tokyo, Japan). The primer pairs used for semi-quantitative RT-PCR were as follows: for Rht-D1a, 5′-ATG AAG CGG GAG TAG CAG GAC G-3′ and 5′-CAT GCC GAG GTG GCC ATG AGC-3′ and for TaCYP20-2, 5′-ATGCGGCCGCCGACCTCCT-3′ and 5′-TACGACCACTGGAAGCTCCCATCC-3′. As a control, a fragment from the gene that encodes T. aestivum alpha-tubulin was amplified by means of the primers 5′-CGTG CAG GGC TCT TG TAC T-3′ and 5′-CAA GGA GTG AGT GGG TGG ACA GGA C-3′. RT-PCR reactions were repeated five times.

For real-time PCR, 2 μg of total RNA was used for RT with Superscripts II reverse transcriptase (Invitrogen). The cDNA samples were diluted to 2 to 8 ng/μL. Triplicate quantitative assays were performed on 5 μL of each cDNA dilution with the SYBR Green PCR Master mix (TaKaRa) and an Mx3000P sequence detection system (STRATAGENE) according to the manufacturer’s protocol (Applied Biosystems). The relative quantification method (ΔΔCT) was used to evaluate quantitative variation between the replicates examined. The amplification of T. aestivum Tubulin was used as an internal control to normalize all data, primers for Tubulin were 5′-TGTGCCCTGTCGTGTCTTATG-3′ and 5′-CCCTTGCCGAGATTGCCCACC-3′. Gene-specific primers for Rht-D1a (GenBank: AI242531) were 5′-GGTTCAAGACCCCTTGCTG-3′ and 5′-CATGAGGGTGGCGGATCACC-3′; for TaCYP20-2 (GenBank: AY217753), 5′-AGCAGGACAGACCTGGTG-3′ and 5′-TACAAGACCTGGAAGCTCCTC-3′; for TaGA20ox1 (GenBank: Y14008), 5′-ATGGTTGACCGCGGTGTTTCAGC-3′ and 5′-GCATGCAGCCGACCCGGATC-3′.

2.5. Protein Sample Preparation and 2D Gel Analysis.

Protein was extracted from aerial part of seedlings of gaid and wild type at 15 day from germination with 9 M urea, 2% CHAPS, 1% DTT and 2% Pharnalyte 3–10, following the method described for 2D electrophoresis manuscript (2D; GE Healthcare). Isoelectric focusing (IEF) for the first dimension was carried out using 7-cm-long IPG strips (GE Healthcare) with a linear pH gradient from 3 to 10. Each sample (600 μL) was mixed with the IEF sample buffer underwent the following steps: 12-h rehydration at 20 °C, 1-h 500 V, 1-h 1000 V, then 8.5-h 8000 V. Intensity was limited to 50 μA/strip. Equilibration of IPG strips was as previously described (GE Healthcare). The first equilibration was performed in a solution containing 6 M urea, 50 mM Tris-HCl, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) bromophenol blue, with 1% (w/v) DTT. The second equilibration was as the first equilibration except that DTT was replaced with 2.5% (w/v) iodoacetamide. SDS-PAGE was carried out at 7 W/gel by use of 12% polyacrylamide gels as described.33 Gels were stained overnight with Coomassie brilliant blue (CBB) G-250. The experiment involved three replicated gels.

2.6. Image and Data Analysis. The CBB-stained gels were scanned on a flatbed scanner (GE healthcare) at 300 dpi resolution. Detection, quantification, and matching of differentially displayed protein spots involved use of ImageMaster 5.0 2D Platinum image software (GE Healthcare). The wild-type and gaid images were compared for 2-DE patterns of protein spots by examining the spot intensity and calculating relative spot volume (%Vol). Only spots showing significant changes between the wild-type and gaid samples (%Vol varied more than 2-fold) were selected for further identification.

2.7. In-Gel Digestion and Mass Spectrometry. All of the differentially expressed spots were manually excised from the 2-DE gels. In-gel digestion and mass spectrometry (MS) acquisition were performed as described.34 The MS spectra were created by use of quadrupole time-of-flight MS (Q-TOF MS; Micromass) equipped with a z-spray source. Tandem MS data were processed by use of MaxEnt 3.0 (Micromass) to create peak lists. The MASCOT version 2.1 search engine (www.matrixscience.com) was used for protein identification by the peptide mass fingerprint search of the National Center for Biotechnology Information (NCBI) database. Carbamidomethyl (C), oxidation (M) and pyro-glu (N-term Q) were set as a fixed modification.

2.8. Immunoblotting. The polycional TaCYP20-2 antiseria was produced as described.35 TaCYP20-2 was amplified for the construction of the prokaryotic expression vector by primers (5′-CGGTATCCATGCGGCACAGAAGCTCCT-3′ and 5′-CGGATATCTCAGACCACCGTCAC-3′). The recombinant plasmid pGEX4T-1-TaCYP20-2 was transformed into Escherichia coli DH5α cells. Polyclonal antisera was raised by subcutaneously inoculating New Zealand white rabbits with about 2.5 mg of recombinant proteins emulsified in an equal volume of Freund’s adjuvant.

IEF for the first dimension was carried out using 7-cm-long IPG strips (GE Healthcare) with a linear pH gradient from 3 to 10. Total proteins were separated by 12% SDS-PAGE and transferred to PVDF membrane. TaCYP20-2 protein was detected by anti-TaCYP20-2 polyclonal antibody. Rht protein was detected by polyclonal antibody raised against the conserved N-terminal domains (DELLA domain and TVHYNPS domain) of barley SLN1. SLN1 from barley is an ortholog of Rht, and has 92% identity with Rht. The α-tubulin polyclonal antibody (Beyotime) was used to detect tubulin protein as loading control.

2.9. Localization of TaCYP20-2-Green Fluorescent Protein (GFP) Fusion Protein. The whole coding sequence of TaCYP20-2 amplified with two primers (5′-CTGAAATGGCGGCACAGAAGCTCCTC-3′ and 5′-GGTACCGACCACCGTCAC-3′) was cloned into the Xbal-KpnI sites of the pBI121 vector to generate pBI121-TaCYP20-2-GFP containing a TaCYP20-2-GFP fusion construct under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter. This construct was electroporated into Agrobacterium tumefaciens C58 and transformed into Arabidopsis by the floral dip method as described.36 Seeds from plants transfected with pBI121-TaCYP20-2-GFP vector were screened on selection medium (1/2 Murishige and Skoog medium, 50 μg/mL kanamycin). The T2 generation was used for further experiments. GFP fluorescence was excited at 488 nm by use of a Confocal microscope (ZEISS LSM 510 META, Zeiss) and detected with 510–550- and 635–680-nm filters.

Leaves from TaCYP20-2-GFP transgenic Arabidopsis were cut into 0.5–1-mm strips with the use of fresh razor blades without wounding. Leaf strips were put in a Petri dish with an enzyme solution (1–1.5% cellulose R10, 0.2–0.4% macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7) and digested for another 60 to 90 min with gentle shaking (40 rpm on a
The enzyme solution turned green, indicated released protoplasts, which were checked under a microscope.

2.10. Generation of TaCYP20-2-overexpressed Transgenic Wheat. TaCYP20-2 was amplified for construction with oligonucleotides 5'-CGGGATCCATGGCGGCCGACCTCCT-3' and 5'-CCGGTACCTCAGACCCAAGCTCCCC-3' used as primers. The PCR fragment was cloned into the pUN1301 vector which carried a β-glucuronidase (GUS) gene as a marker. The TaCYP20-2 gene was driven by the ubiquitin promoter. This construct was verified by sequencing and transformed into the
Table 1. Heterosis of Plant Height over the Parents*

<table>
<thead>
<tr>
<th>male parent (♂)</th>
<th>height of male parent (cm)</th>
<th>height of F₁ (cm)</th>
<th>reducing effect (%)</th>
</tr>
</thead>
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<tr>
<td>ND3214</td>
<td>80 ± 1.5</td>
<td>70 ± 1.4</td>
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</tr>
<tr>
<td>ND99–5009</td>
<td>105 ± 0.8</td>
<td>72 ± 1.4</td>
<td>31.4%</td>
</tr>
<tr>
<td>Jing411</td>
<td>90 ± 1.4</td>
<td>75 ± 1.3</td>
<td>16.7%</td>
</tr>
<tr>
<td>BAU33338</td>
<td>55 ± 1.4</td>
<td>60 ± 1.0</td>
<td>−9.1%</td>
</tr>
<tr>
<td>gaid</td>
<td>60 ± 1.1</td>
<td>60 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

*a gaid mutant was used as female parent. Beijing local varieties of winter wheat DNS3214, ND99–5009, Jing411 and BAU3338 were used as male parents. ND means Nong Da. Results are presented as means ± SE.

3. Results

3.1. Genetic Effects of gaid Mutant on the Height of Wheat Varieties. A wheat mutant with dwarfism was screened from a natural population of variety wheat, Jingdong No.1. After 5 generations of selection, we established a wheat line with a dwarf phenotype during all growth stages from seedling to maturation as compared with the wild-type (Figure 1A–D,F). Leaves, leaf sheathes and stem in gaid were shorter than that in the wild type. The height-reduced percentage of gaid mutant was up to approximately 25% of the wild type from the seedling stage to heading. Numbers of the reduced percentage were quite close among leaves, leaf sheathes, and stem. To explore the genetic characters of gaid, the fifth generation plant as a female parent was crossed with the local varieties ND3214, ND99–5009, Jing411, as well as BAU3338, a dwarf mutant, as a male parent. The results showed a clear phenotype in height in progenies (Table 1). The height of most F₁ hybrid progenies of gaid mutant was lower than that of the local varieties (ND3214, ND99–5009, Jing411) and was 60–75 cm. In contrast, the height of the F₁ hybrid progenies of gaid and BAU3338 was higher than that of the male parent BAU3338 but was identical to that of their female parent, gaid. The similar thousand-grain weight of the wild type (50–60 g) and gaid plants (55–65 g; data not shown) indicates that the yield of gaid mutant was not negatively influenced.

3.2. Physiological Effect of GA₃ on gaid Growth. To elucidate the molecular mechanism of gaid, we tested the response of gaid to exogenous GA₃ treatment. Fifteen-day-old seedling of wild-type wheat responded positively to GA₃ treatment in shoot elongation (Figure 1A,C), and the length of the first leaf sheath increased by 57.4% (from about 4.7 to 7.4 cm). In contrast, in gaid, the length of the first leaf sheath had no significant difference compared with those of the same treatment (P > 0.05; Figure 1A,C).

As known, from the vegetative to reproductive stage, wheat internode elongation occurs only at the shoot apical meristem with exogenous GA₃ treatment. Figure 1B and C shows that the application of GA₃ did not result in internode elongation in gaid. In contrast, in the wild type, all of the first 5 internodes were elongated with exogenous GA₃ treatment, and shoot height increased from 90 to 110 cm. These results suggested that gaid was insensitive to GA₃ treatment.

To determine whether the reduction of shoot elongation in gaid was caused by either decreased cell elongation or cell number, epidermal layers of the first leaf sheath (approximately 5-mm of apex segment) were examined under a differential interference contrast microscope. Under the normal condition, the length of epidermal cells in the first leaf sheath of the wild type (388.6 μm) was much greater...
than that of gaid (289 µm; Figure 1H,F,J). After GA3 treatment with 100 µM for 24 h, the mean length of epidermal cells of the wild type was increased by 45%, to 562.3 µm (Figure 1I,J). But the effect of the GA3 treatment on epidermal cells in gaid (316.4 µm; Figure 1G,J) did not reach a level with statistical significance (P > 0.05).

The gaid plants are more insensitive to PAC, a GA biosynthesis inhibitor, than the wild type. Dose–response results with PAC treatment (Figure 1E) showed that the first leaf sheath length of the wild type was inhibited at 10⁻⁸ M. At 10⁻⁷ M of PAC, the wild type was dwarfed similar to gaid. In contrast, the length of the first leaf sheath in gaid did not change with PAC treatment up to 10⁻⁷ M. At a higher concentration of PAC (>10⁻⁷ M), the first leaf sheath length in both gaid mutant and the wild type was reduced to the same level.

3.3. Expression Patterns of TaGA20ox1 and Rht in gaid.

As known, mutants with impaired response to GAs have always elevated GA biosynthesis genes expression and reduced DELLA protein turnover.¹⁸ To investigate whether transcriptional pattern on genes in GA biosynthesis was changed in gaid, we monitored the transcript level of TaGA20ox1 gene. The results revealed that treatment of the wild type with GA3 for 3 h induced a drastic reduction in mRNA level of TaGA20ox1 (Figure 2A). However, the transcript level of TaGA20ox1 in gaid increased slightly after GA3 treatment. The expression pattern response of TaGA20ox1 in Rht-1 wheat mutant was lightly elevated with GA3 treatment (Figure 2A), which was similar to that in the gaid mutant. In Rht-B1a wheat allele as the wild type, contrarily, a significant decrease transcript level of TaGA20ox1 appeared after GA3 treatment.

The gain-of-function mutation of Rht can suppress the effects of GA in wheat with phenotypes of dwarfism and GA-insensitivity.² The analysis of the Rht-D1a gene in the genome of gaid mutant indicated that the open reading frame sequences was the same as wild type (data not shown). Real-time PCR results showed that mRNA level of Rht-D1a was similar between gaid and wild-type plants whether with GA3 treatment or not (Figure 2B). The Rht protein expression pattern on immunoblotting assay (Figure 2C) showed that its level decreased after GA3 treatment in the wild type. In gaid mutant, on the contrary, any response of the Rht protein level to GA3 on degradation was not detected. As a typical DELLA mutant response, the expression pattern response of Rht in Rht-1 wheat mutant was not decrease after the treatment of GA3 (Figure 2D). Contrarily, Rht protein decreased significantly with GA3 treatment in its wild type plants. It suggested that the degradation of Rht induced by GA was suppressed in gaid mutant.


A comparative proteomic approach was used to explore the mechanism of dwarfism in gaid. Total aerial part proteins extracted from gaid
and the wild type were separated by 2-DE. The representative 2-DE leaf protein maps are shown in Figure 3A. ImageMaster analysis of at least three independent gels revealed about 500 protein spots reproducibly resolved on Coomassie-stained gels over a combined pH of 3–10 and molecular weight (MW) 6–120 kDa in gaid or wild type. Although more than 99% of the protein spots remained with consistent expression levels in gaid and the wild type, 5 protein spots (name as spot 1 to spot 5; Figure 3A) were reproducibly detected as changed in their protein levels. Figure 3B showed the Venn diagram analysis of these differentially expressed spots at various samples. Both spot 1 and spot 2 were down-regulated, and contrarily, spot 3 and spot 4 were up-regulated in gaid as compared with the wild type. There was no changed pattern in spot 5 in the mutant compared with the wild type. After GA3 treatment, spot 5 was accumulated more in gaid than that in the wild type. Of note, spot 4 could still not be observed in the wild type under GA3 treatment on Coomassie-stained gels. Moreover, in gaid, spot 4 was not significantly accumulated after the treatment (Figure 3C,D). The expression pattern of spot 4 in gaid was independent of GA3 treatment.

Differentially expressed proteins were excised from the gels, in-gel digested by trypsin, and identified by a Q-TOF MS. The results showed that spot 1 and spot 3 were characterized as a predicted and a hypothetical protein respectively. Spot 2 was identified as ATP synthase CF1 alpha chain. Spot 4 and spot 5 were revealed corresponding to wheat cyclophilin family proteins. Spot 5 matched a cyclophilin A-1 protein, and spot 4 corresponded to a cyclophilin-like protein (TaCYP20-2) (Table 2).

Table 2. Identification of Differentially Induced Proteins in gaid Mutant

<table>
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<th>matched peptides</th>
<th>Mr</th>
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<td>2</td>
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<td>NP_039282</td>
<td>66</td>
<td>Marchantia polymorpha</td>
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<td>55.3</td>
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<td>4</td>
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<td>5</td>
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<td>AAK49426</td>
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Figure 4. Immunoblot analysis of TaCYP20-2 expression in gaid mutant. (A) The specificity test of polyclonal TaCYP20-2 antibody. Purification of GST-TaCYP20-2 recombinant protein (lane 1) and leaf protein extracted from wheat (lane 2) were separated by 12% SDS-PAGE and detected by polyclonal TaCYP20-2 antibody. Lane 3 and lane 4 were the Coomassie brilliant blue (CBB)-stained GST-TaCYP20-2 recombinant protein and whole leaf protein as a loading control. (B) Loading control of immunoblot analysis of TaCYP20-2 protein level (C) in the wild type (lane 1), gaid mutant (lane 2), the wild type treated with GA3 (lane 3), and gaid mutant treated with GA3 (lane 4). (C) Leaf protein (80 µg) of wild type, gaid mutant, the wild type treated with GA3, and gaid mutant treated with GA3 were separated by 2-DE and detected by polyclonal TaCYP20-2 antibody. (D) %Vol of detected spot 7.8 by immunoblotting. (E) %Vol of detected spot 8.0 by immunoblotting.
3.5. Confirmation of the Accumulation of TaCYP20-2 Protein in gaid.

To confirm the results of 2-DE and MS, the protein level of TaCYP20-2 in gaid was monitored by immunoblotting. The specificity of the antibody raised against TaCYP20-2 was detected first. Theoretically, TaCYP20-2 is 25.9 kDa. Two stronger bands at 52 kDa and 26 kDa were recognized in the blot by the antibody in purified the GST-tagged recombinant TaCYP20-2 protein (Figure 4A). In the whole protein extract of the wild-type wheat, the immunoblotting showed the antibody with specificity to a 26-kDa protein, which was consistent with TaCYP20-2 (Figure 4A).

The protein extracts from the aerial part of the wild type and gaid, as well as the treated ones, were separated by 2-DE with IEF (pH 3–10; 7 cm) for the first dimension and 12% SDS-PAGE for the second dimension. Then the four gels were blotted and detected by the antibody raised against TaCYP20-2. The immunoblotting with 2-DE gels (Figure 4C) showed two TaCYP20-2 isoforms with different pI values (8.0 and 7.8). Since the observed pI values were different from the theoretical one (pI 9.4), we assumed that the discrepancy between its observed pI values and theoretical one resulted from an acidic shift, and the two protein spots (spot 8.0 and spot 7.8) represented the different post-translational modification of TaCYP20-2. The observed pI value of spot 4 was also 7.8. The kinetics of protein-abundance change of spot 7.8 determined by immunoblot (Figure 4C,D) was very similar to that measured by 2-DE (Figure 3C,D). Spot 7.8 was up-regulated in gaid compared with that in the wild type (Figure 4C,D). In gaid, the protein level of spot 8.0 was also slightly higher than that in the wild type (Figure 4C,E). These results confirmed TaCYP20-2 was accumulated in gaid compared with that in the wild type.


The TaCYP20-2 gene contains an open reading frame of 245 amino acids, with molecular mass 25.9 kDa and pI 9.40. TaCYP20-2 protein is constituted by only a single cyclophilin ABH domain, which is the core domain of the cyclophilin family.39,40 To explore the relation between TaCYP20-2 and other single-domain cyclophilins, a sequence-based phylogenetic tree was constructed (Supplementary Figure 1A in Supporting Information). The polygenetic tree consists of three major groups. TaCYP20-2 and TaCYP20-c are different copies of the same gene in hexaploid wheat. Rice protein NP_001054392 is an ortholog of TaCYP20-2, which showed the highest identity (82%) with TaCYP20-2 protein. The Arabidopsis ortholog with the highest similarity (67%) is AtCYP20-2. Alignment analysis showed that TaCYP20-2 gene shared higher sequence similarity with single-domain cyclophilins from rice, Arabidopsis, maize, tomato, bean and human (Supplementary Figure 1B in Supporting Information).

The mRNA level of TaCYP20-2 in various wheat tissues was detected by semiquantitative RT-PCR (Figure 5A). The result indicated that the transcript of TaCYP20-2 was constitutive of expression in various tissues. Preponderant expression appeared in young leaves. The transcriptional time course in response to GA3 in the wild type showed that the TaCYP20-2 mRNA level increased rapidly within 24 h under GA3 treatment (Figure 5B). Subsequently, the mRNA level began to decline up to 24-h treatment. These results indicated that the mRNA...
level of TaCYP20-2 was induced by GA3, which is consistent with the data on protein level described above (Figure 4C,D).

Real-time PCR analysis revealed that mRNA level of TaCYP20-2 in gaid mutant wheat was slightly higher than that in the wild type (Figure 5C). After GA3 treatment, the TaCYP20-2 mRNA level declined to the similar level between the wild type and gaid mutant.

3.7. Subcellular Localization of TaCYP20-2:GFP Fusion Protein. To examine its subcellular localization, TaCYP20-2 driven by the ubiquitin promoter was transformed into wheat plants to create three T0 generation lines of transgenic plants. The transformation lines were screened by hygromycin as well as genomic PCR (data not shown). TaCYP20-2 transgenic wheat (Figure 7) was confirmed by semiquantitative RT-PCR. TaCYP20-2 was detected in the wild-type plants, but the transgenic line showed a much higher expression level (Figure 7C). The immunoblotting result showed the protein level of TaCYP20-2 was increased significantly in transgenic lines, such as L1, L2 and L3, which agrees with the results of RT-PCR (Figure 7D). The changed protein expression pattern of Rht was detected by immunoblotting in TaCYP20-2 overexpressed transgenic wheat lines. The result indicated Rht protein accumulated higher in all three TaCYP20-2 transgenic lines than that in the wild type (Figure 7D and E).

Table 3. Height (cm) of the Wild-Type and T1 Transgenic Wheat after Heading

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<th>Wild type</th>
<th>Transgenic Line1</th>
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<td></td>
<td>50.12 ± 5.47</td>
<td>39.34 ± 2.66</td>
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*Results are presented as means ± SE.

To examine the possible phenotypes of transgenic wheat, T0 and T1 generations of TaCYP20-2-overexpressed wheat, as well as the wild-type plants, were grown in the field after vernalization for 30 days. Compared with the wild-type plants, the transgenic wheat showed a clear dwarf phenotype during all growth stages from seedling (Figure 7A) to maturation (Table 3), which was similar to the dwarf phenotype of gaid. The length of the first leaf sheath of TaCYP20-2-overexpressed wheat was less than that of the wild type at the seedling stage (Figure 7B). After heading, the height of TaCYP20-2-overexpressed plants reached approximately 75% of that of the wild type (Table 3). The difference in height between the wild type and TaCYP20-2-overexpressed wheat was statistically significant (P < 0.05).

4. Discussion

4.1. The gaid Dwarf Mutant is a New Variety for “Green Revolution” Breeding. In grain crops, dwarfism of wheat and rice cultivars is a major factor for the success of the “Green Revolution”. For instance, sd1 semidwarf rice and Rht semi dwarf wheat have been widely utilized in crop improvement for higher grain yield. In this study, we characterized a novel dwarf wheat mutant, gaid. The semidominant dwarf phenotype of gaid could be stably inherited. Except for the dwarf mutant BAU3338, the local cultivars showed reduced height after hybrid with gaid (Table 1). The progenies with reduced height retained the same seed weight as that of their parents. Therefore, gaid could be a good variety candidate for “Green Revolution”
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because of its moderate dwarfism in relation to stem growth and panicle development.

The dwarf phenotype of gaid with treatment of GA3 can not be rescued (Figure 1). Plants of gaid responded less to GA3 than wild type in terms of the first leaf sheath elongation. Moreover, gaid mutant plants responded to PAC with a concentration 10 times higher than the wild type did, which indicates that dwarfism of gaid mutant is independent of endogenous GA level at normal physiological status. The wheat Rht mutant (Rht-B1b and Rht-D1b) also showed only a slight growth response to GA3, even at high concentration.2 The length of leaf sheath epidermal cells in gaid is much shorter than the wild type (Figure 1). It indicates that the dwarfism of gaid resulted from reduced cell elongation. The Rht-B1b and Rht-D1b GA-insensitive dwarfing genes are able to decrease cell wall extensibility to reduce epidermal cell length.3 Therefore, it is possible that the shortened leaf sheath cell length in gaid resulted from up-regulated Rht gene expression. Changed expression of GA biosynthesis genes by GAs induction has been described in several GA-insensitive mutants.42 GA 20-oxidase is a primary target for feedback regulation. As known, the gain-of-function mutations in the repressors or loss-of-function mutations in the positive components of the GA response pathway often lead to an up-regulation of GA20ox gene expression.42 For example, GA20ox gene mRNA level decreased in the wild type Arabidopsis after GA3 treatment, while the level increased slightly in the gai mutant.11,38 The changed patterns of TaGA20ox1 transcript level under GA3 treatment in gaid and Rht-1 (Figure 2) are comparable to above phenomenon of Arabidopsis. Treating the plants with GA3 caused TaGA20ox1 mRNA level declining in the wild type. In contrast, the level increased slightly in gaid and Rht-1. However, our data on GA20ox1 expression patterns showed that the basal expression level of TaGA20ox1 either in gaid or Rht1 mutant was lower that in their relative wild type plants, respectively. It is possible that others GA oxidases, such as GA20ox-2, GA2ox and GA3ox, may respond in the mutants. At a minimum, there are two GA 20-oxidase genes (GA20ox-1 and GA20ox-2) and SD1 corresponded to GA20ox-2 in the rice genome. GA20ox2 is highly expressed in leaves and flowers, whereas GA20ox1 is preferentially expressed in the flowers.43 Mutants with impaired responses to GA have always elevated GA biosynthesis genes expression and reduced DELLA protein turnover.38 The DELLA protein Rht was accumulated in gaid mutant similar to Rht-1 wheat mutant (Figure 2). These phenotypes of gaid suggest that it is due to mutations in a gene participating in a GA signaling pathway rather than a GA biosynthesis pathway. The sequence of the Rht gene was conservative in genome of the gaid mutant (data not shown). These evidence as well as the physiological ones on the response to GA3 suggest that gaid is a new semidwarf wheat mutant in the GA signaling pathway through a different mechanism from that of published mutants, including Rht and Snl.

4.2. Accumulation of TaCYP20-2 Causes Dwarf in Wheat.

In this work, comparative proteomics as a powerful quantitative experimental strategy is first applied for investigating wheat mutant. Using this approach, we identified some regulated proteins as candidates for proteins with important roles in gaid. Most convincingly, we found the protein level of TaCYP20-2 was accumulated in gaid (Figure 3). Immunoblotting provided further evidence similar to this result of 2-DE (Figure 4). On the basis of Real-time PCR results (Figure 5), we considered that the higher transcript level of TaCYP20-2 gene resulted in the accumulation of TaCYP20-2 protein in gaid. Because wheat genome is a hexaploid, most allele genes are of functional redundancy. Almost all of the wheat mutants are resulted from dominant gain-of-function mutations. Accordingly, we propose a hypothesis that the accumulation of TaCYP20-2 should be associated with the dwarf phenotype in gaid.

TaCYP20-2 possesses all of the key structural features of the cyclophilin family (Supplementary Figure 1 in the Supporting Information). In human cyclophilin A, Trp-121 was identified as a residue required for CsA binding but not participated in PPIase enzymatic activity by mutagenesis studies.44,45 Moreover, mutations in Arg-55, Phe-60 and His-126 result in PPIase enzymatic activity reduced to 1% of original human cyclophilin A activity.45 Therefore, corresponding conservative amino acid residues in TaCYP20-2 may play vital roles. TaCYP20-2 was predicted to be targeted to the chloroplast and had no transmembrane region by the neural-network-based targeting prediction program TargetP (data not shown). However, subcellular localization analysis results indicated that the TaCYP20-2-GFP fusion protein accumulated mainly in the chloroplast and cell plasma membrane (Figure 6). Expression-pattern analysis showed TaCYP20-2 is in various tissues of wheat (Figure 5). The results suggest that TaCYP20-2 protein was not only restricted to the photosynthetic tissue but also attached to the cell membrane. On the basis of this evidence, it is possible that TaCYP20-2 plays a role during signaling transduction.

Previous reports have indicated that cyclophilins have a great influence on the sensing and signaling pathways of plant hormones. LeCYP1, a tomato type-A cyclophilin highly homologous to human cyclophilin A, is induced by auxin treatment and essential for morphogenesis of lateral root primordial.46 According to that, FKBP42 is present in the plasma membrane and interacts with the multidrug resistance/P-glycoprotein (PGP); FKBP42 is proven to play a positive regulatory role in PGP-mediated export of auxin.47 Studies of the Arabidopsis pas1 mutant indicated that FKBP70 was involved in cell division through cytokinin signaling pathway.28 Accumulation of the heat-stress protein FKBP77 in wheat also has a negative effect on plant development, especially causing dwarfism.38 These evidence raise the possibility that the accumulation of TaCYP20-2 may cause dwarfism of gaid through GA signaling pathway. Consistent with gaid mutant wheat, TaCYP20-2-overexpressing transgenic wheat showed a 25% shorter stem than the wild type from seedling to mature stage (Figure 7 and Table 3), which indicates that TaCYP20-2-overexpressed transgenic wheat displays a dwarf phenotype. These findings indicate the accumulation of TaCYP20-2 would induce dwarfism in wheat.

4.3. Degradation Suppression of DELLA Protein Mediated by the Accumulation of Cyclophilin is Involved in gaid Mutant.

The gaid mutation resulted in the semidominant dwarf blocking of the GA signaling pathway, a phenotype analogous to that of the gain-of-function mutation of Rht in wheat2 and GA1 in Arabidopsis.11 The transcript levels of Rht-D1a did not change in gaid seedlings with or without GA3 treatment, similar to that in wild-type (Figure 2B). Rht-D1a gene in gaid genome showed no mutation at DNA sequence as compared with the wild type. There is a great probability that the accumulation of Rht protein leads to gaid mutant. In wild-type plants, bioactive GAs can depress the action of negatively acting components such as GAI/SLR/SLN/RHT and regulate the degradation of these repressor proteins during seed germina-
tion and shoot growth.\textsuperscript{19} Rht protein in \textit{gaid} did not respond to GA\textsubscript{3} treatment, whereas it declined significantly after GA\textsubscript{3} treatment in the wild-type plants (Figure 2C). Remarkable up-regulation of Rht protein level had been observed in the transgenic wheat lines (Figure 7D and E). These results strongly support the idea that there is a positive correlation between the protein levels of Rht and TaCYP20-2. The protein level of TaCYP20-2 in \textit{gaid} mutant is about three times higher than that in the wild type. The difference of Rht protein between \textit{gaid} mutant and the wild type without GA\textsubscript{3} treatment is less evident in the immunoblotting analysis. However, in the transgenic wheat, TaCYP20-2 level is dozens of times higher than that in the wild type. Meanwhile, the protein change trends of TaCYP20-2 and Rht in different transgenic wheat lines are consistent. It suggests that Rht degradation may be suppressed in \textit{gaid} mutant. It becomes clear that the dwarfism of \textit{gaid} did not result from the mutation in \textit{Rht-D1a} gene, but, rather, the accumulation of Rht protein. Taken together, the TaCYP20-2-overexpressed transgenic wheat showed a higher level of Rht protein and reduced plant height. These properties are also characteristics of \textit{gaid} mutant, indicating that \textit{gaid} mutant is caused by the accumulation of TaCYP20-2 protein. On the basis of the evidence that the transcription of TaCYP20-2 was increased in the \textit{gaid} mutant (Figure 5C) and there was not any difference on the gene sequences between the mutant and wild type (data not shown), it is possible that there is an unknown element to stimulate the TaCYP20-2 transcription on genome resulting in the dwarf mutant.

The research on the cyclophilin-like domain of Ran-binding protein-2 (RanBP2) in human cells revealed that it selectively promoted the accumulation of properly folded targets of the ubiquitin-proteasome system for degradation.\textsuperscript{49} It is well-known that DELLA proteins are targets of the ubiquitin-proteasome system.\textsuperscript{17,18} The expression level of Rht increased in the transgenic wheat compared with the wild type (Figure 7). Combined with previous results that the degradation of Rht protein was repressed in \textit{gaid} (Figure 2), it is reasonable to deduce that the accumulation of TaCYP20-2 causes an increase in Rht protein abundance and leads to dwarfism phenotype of \textit{gaid} according to the gain-of-function mutation of Rht in wheat. A cyclophilin protein was identified as a peptidyl-prolyl cis-trans isomerase (PPlase) in spinach. It catalyzes the key step for protein folding, which was shown in the thylakoid lumen.\textsuperscript{23} The selective effect of cyclophilin protein on the ubiquitin-proteasome system is a synergy with proteasome inhibitors or factors to suppress activity of the 26S proteasome. The factor(s) may be necessary to mediate the post-translational modification and to inhibit its recognition by a pool of ubiquitinated substrates.\textsuperscript{49} Like that in human cells, it is possible the cyclophilin-like protein selectively modulates the activity of the ubiquitin-proteasome system through its interaction with inhibitor(s) to regulate the turnover of properly folded proteins which are essential for plant development. Such a scenario might explain, at least in part, the intriguing roles that TaCYP20-2 appears to play as a negative regulator in GA signaling pathway through modulating DELLA protein abundance. GAID/CYP20-2 as a PPIase may be involved in protein modulation and/or protein folding to impact proteasome system. It should be substantiated with further experimental evidence in the future.

5. Conclusions

This report has characterized a novel dwarf wheat mutant, \textit{gaid}, which is a very useful genetic source for conventional hybrid breeding and deep understanding of the mechanism of GA signaling pathway. Furthermore, we first provide comparative proteomic and immunoblotting evidence to reveal TaCYP20-2 enriched in \textit{gaid}. This, combined with the discovery of TaCYP20-2-overexpressed transgenic wheat exhibiting a dwarf feature similar to that of \textit{gaid} mutant wheat, suggested that the accumulation of TaCYP20-2 is involved in the regulation mechanism of dwarfism in the \textit{gaid} mutant. As known, the cell extension for growth is controlled by the signaling pathway of GAs-mediated DELLA protein degradation. Our data on the relation between cyclophilin and DELLA protein indicates that TaCYP20-2 is a new negative regulator in the GA signaling transduction pathway of DELLA protein degradation for cell extension in nature case. This report provides beneficial information for molecular breeding leading to “Green Revolution” of agricultural crops.

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Supporting Information Available: Supplementary Figure showing phylogenetic tree of cyclophilin proteins from different organisms (A), and alignment of the amino acid sequence for TaCYP20-2 and sequences encoded by related genes of wheat, rice, Arabidopsis, maize, tomato, fava bean and human (B). This material is available free of charge via the Internet at http://pubs.acs.org.

References

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