Regulation of Expansin Gene Expression Affects Growth and Development in Transgenic Rice Plants

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To investigate the in vivo functions of expansins, we generated transgenic rice plants that express sense and antisense constructs of the expansin gene OsEXP4. In adult plants with constitutive OsEXP4 expression, 12% of overexpressors were taller and 88% were shorter than the average control plants, and most overexpressors developed at least two additional leaves. Antisense plants were shorter and flowered earlier than the average control plants. In transgenic plants with inducible OsEXP4 expression, we observed a close correlation between OsEXP4 protein levels and seedling growth.coleoptile and mesocotyl length increased by up to 31 and 97%, respectively, in overexpressors, whereas in antisense seedlings, they decreased by up to 28 and 43%, respectively. The change in seedling growth resulted from corresponding changes in cell size, which in turn appeared to be a function of altered cell wall extensibility. Our results support the hypothesis that expansins are involved in enhancing growth by mediating cell wall loosening.

INTRODUCTION

Plant cell walls function as structural support, determine cell shape, and protect the cell against external biotic and abiotic stress. To grow, plants must loosen their rigid cell walls in a precisely controlled spatial and temporal pattern. The expansin proteins, discovered by McQueen-Mason et al. (1992), are prime candidates for cell wall-loosening factors that mediate the growth of plant cells (reviewed by Cosgrove, 2000; Cosgrove et al., 2000). They were shown to cause stress relaxation of isolated cell walls in a pH-dependent manner, and the prevailing model posits that expansins act by disrupting hydrogen bonds between cellulose microfibrils and matrix polysaccharides or between matrix polysaccharides themselves (McQueen-Mason and Cosgrove, 1994; reviewed by Cosgrove, 2000).

The role of expansins as mediators of plant cell growth has been tested by a number of approaches. In many instances, a close correlation has been found between growth and the presence and activity of expansin proteins (McQueen-Mason et al., 1992; Wu et al., 1996; Cho and Kende, 1997b). There also is mounting evidence for a close correlation between growth and the expression of expansin genes (reviewed by Lee et al., 2001; Cosgrove et al., 2002). In cases in which such correlations are lacking, it has been suggested that some expansins may play a role in cell differentiation rather than in cell growth (Caderas et al., 2000; Reidy et al., 2001). Application of expansin preparations at different stages of purification was shown to promote the growth of suspension-cultured tobacco cells (Link and Cosgrove, 1998) and excised Arabidopsis hypocotyls (Cosgrove et al., 2002), to cause the bursting of cucumber root hairs (Cosgrove et al., 2002), and to induce the formation of leaf-like structures on the shoot apical meristem of tomato plants (Fleming et al., 1997). However, no pure recombinant expansin has been available to test its activity rigorously by application to live plant tissues or isolated cell walls.

The role of expansins in growth and cell wall modifications also has been tested by manipulating the expression of expansin genes in transgenic plants. Brummell et al. (1999) investigated the role of expansin in the ripening process of tomato fruits by either enhancing or suppressing the expression of an expansin gene. Endogenous expansin was shown to affect cellular wall metabolism in the fruit and to enhance fruit softening. However, the average size of fruits with low expansin accumulation was not different from that of control fruits, whereas fruits with increased expansin content were on average half the size of control fruits. The height of plants that overexpressed the expansin gene was not affected, although their leaves were slightly smaller. Rochange et al. (2001) also tested the role of endogenous expansin in tomato plants by overexpressing an expansin gene. Three independent transgenic lines with high expansin activity were evaluated in detail. The growth of both mature plants and etiolated seedlings was reduced severely, as were the cell size and cell wall extensibility of dark-grown hypocotyls. Expansins have been identified in the bryophyte Physcomitrella patens, in which null mutants can be obtained by homologous recombination (Schipper et al., 2002). Four expansin genes were mutated successfully by this method, but no change in phenotype was observed in any mutant that lacked expression of an expansin gene. None of these experiments with transgenic plants provided support for the hypothesis that expansins are involved in mediating the growth of whole plants, plant organs, or plant cells. The lack of phenotypic alteration in

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.011965.
plants with reduced expansin gene expression may be explained by the functional redundancy of expansin proteins (Schipper et al., 2002). However, it is more difficult to explain the lack of effect or the inhibition of growth in plants that over-express an expansin gene. Reduced sensitivity of transformants to expansins was cited as one possible cause (Rochange et al., 2001).

In contrast to the results described above, two experimental approaches—both involving the growth and development of leaves—lent support to the expansin hypothesis. Transgenic Arabidopsis plants expressing sense or antisense constructs of the α-expansin cDNA AtEXP10 exhibited increased or decreased growth of petioles, respectively (Cho and Cosgrove, 2000). Pedicel abscission was enhanced in sense plants and retarded in antisense plants. Using a microinduction system, Pien et al. (2001) demonstrated that local expression of an α-expansin gene initiated leaf development and altered leaf shape.

Expansins are not the only candidates for endogenous cell wall-loosening factors that mediate plant growth. Because of the ability of hydroxyl radicals (HO·) to cleave polysaccharides, Fry (1998) investigated whether HO· was capable of cleaving cell wall polysaccharides. He found this to be the case under conditions that may prevail in the cell wall. Therefore, Fry suggested that HO· might mediate cell wall loosening and questioned whether enzymes and expansins are the only cell wall-loosening factors. The potential role of HO· in cell wall loosening and elongation growth was investigated by Schopfer (2001) and Schopfer et al. (2002). It was shown that HO· promotes the extension of isolated cell walls just as expansin does; that experimental generation of HO· in live coleoptile and hypocotyl sections induces elongation growth; that coleoptiles produce HO·; that auxin causes the release of reactive oxygen intermediates into the cell wall; and that HO· scavengers inhibit the auxin-induced growth of coleoptile sections. Schopfer (2001) and Schopfer et al. (2002) proposed that HO· is generated from O₂⁻ and H₂O₂ by cell wall-localized peroxidases and that HO· rather than expansins serves as a wall-loosening factor.

In previous work, we isolated and characterized an α-expansin cDNA (OsEXP4) of deepwater rice and showed that OsEXP4 gene expression was induced in the intercalary meristem within 30 min of gibberellin (GA) treatment or submergence of the plant (Cho and Kende, 1997c). This increase in OsEXP4 transcript level occurred before a GA- or submergence-induced increase in the growth rate of the internode could be measured. Based on this result, we proposed that OsEXP4 plays a role in the submergence- and GA-promoted stem elongation of deepwater rice. Expansins also are thought to play a role in the growth of rice seedlings (Huang et al., 2000). Of the α-expansin gene family of rice, OsEXP1, OsEXP2, OsEXP4, and OsEXP10 are highly expressed in growing coleoptiles (Cho and Kende, 1997c; Lee and Kende, 2002). When seeds were germinated and seedlings were grown under submerged or hypoxic conditions, coleoptile growth was promoted strongly and OsEXP2 and OsEXP4 mRNA accumulated in the coleoptile (Huang et al., 2000).

As a first step in analyzing the in vivo function of expansins in rice, we transformed rice with sense and antisense constructs of OsEXP4 cDNA under the control of the constitutive ubiquitin (Ubi-1) promoter and observed the effects of increased and reduced expansin gene expression on the growth and development of adult rice plants. To investigate the function of OsEXP4 at specific developmental stages or during the growth of specific organs of rice, we adopted the maize In2-2 promoter system, which is inducible with the herbicide safener N-(aminocarbonyl)-2-chlorobenzenesulfonamide (2-CBSU) (Hershey and Stoner, 1991; Veylder et al., 1997; Boetti et al., 1999). Here, we show the effects of sense and antisense expression of OsEXP4 cDNA on the growth of rice seedlings and adult rice plants and provide evidence that endogenous OsEXP4 plays an important role in mediating the growth and development of rice plants.

RESULTS

Constitutive Expression of Sense and Antisense OsEXP4 Constructs in Rice Plants Yields Pleiotropic Phenotypes

To determine the role of endogenous expansin in the growth and development of intact rice plants, we transformed rice embryogenic calli with constructs containing OsEXP4 cDNA in the sense (pSUBGX4S) and antisense (pSUBGX4As) orientations and with the vector alone as a control (pSUBG), all under the control of the constitutive Ubi-1 promoter (Figure 1A). We obtained 79 independent T₀ regenerants: 18 plants harboring the vector alone (control), 37 plants harboring the sense construct, and 24 plants harboring the antisense construct. The presence of the transgenes was confirmed by genomic DNA gel blot analysis (Figures 2A and 2B). Some transformed lines carried more than one copy of the transgene (Figure 2B). Expression of the transgenes was detected by RNA gel blot analysis performed with total RNA extracted from mature leaves of transgenic plants (Figures 3A and 3B). Mature leaves do not express the OsEXP4 gene (Cho and Kende, 1997c); therefore, they are a suitable organ in which to determine the expression of the introduced OsEXP4 constructs. All plants for which the presence of the transgene was confirmed by DNA gel blot analysis expressed the transgene transcripts in mature leaves (Figures 3A and 3B).

Overexpression of OsEXP4 yielded pleiotropic phenotypes in plant height, leaf number, flowering time, and seed set. Transgenic sense plants segregated into two subpopulations that were either taller or shorter than the average control plant (Figure 4A). Plants taller than the average control plant (78 cm) had a height of 85 to 95 cm, whereas those shorter than the average control plant fell into the range of 30 to 75 cm (Figure 5A). The sense transgenic plant S-24 (Figure 4A, plant d) was one of the short sense plants, had multiple copies of the transgene (Figure 2B), and had the highest level of transgene expression (Figure 3A). Transgenic plant S-4 was one of the taller overexpressing plants (Figure 4A, plant c), with a single copy of the transgene (Figure 2B) and an intermediate level of transcript expression (Figure 3A). In general, shorter transgenic sense plants (S-21 to S-25; Figure 3A) contained higher levels of OsEXP4 transcripts than did taller transgenic sense plants (S-3 to S-6; Figure 3A). Most transgenic sense plants developed at least two more leaves than did control plants, regardless of how many copies of the transgene they harbored (Figure 5B). Some transgenic sense plants had multiple secondary tillers.
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at the first node and abnormally elongated leaf sheaths, and some had a secondary branch on the second or third node (Figure 4B). Flowering in transgenic sense plants was delayed by 2 to 4 weeks compared with flowering in control plants (Figure 5C). All transgenic sense plants flowered but were sterile, whereas most control and antisense plants developed viable seeds. Fertility of sense transformants was not recovered in ratooned plants grown under the same culture conditions (data not shown). At flowering is the growth of a new shoot from the root stock after the original shoot has been harvested) (results not shown). Antisense lines had one or two copies of the transgene (Figures 2A and 2B) and showed relatively low transcript levels (Figure 3B). They did not exhibit morphological abnormalities, but most of them were shorter than control plants, and all flowered up to 3 weeks earlier than did control plants (Figures 4A and 5A to 5C). The same effect was observed in ratooned plants grown under the same culture conditions (data not shown).

Growth of Transgenic Seedlings Is Correlated with the Level of OsEXP4 Protein

Genes under the control of the In2-2 promoter are inducible with the herbicide safener 2-CBSU. We determined the optimal

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<tr>
<th>Figure 1. Structures of Constructs for Rice Transformation.</th>
<th>Figure 2. DNA Gel Blot Analysis of Genomic DNA from Adult Transgenic Plants.</th>
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<td>(A) OsEXP4 constructs under the control of the constitutive Ubi-1 promoter. The phosphinothricin acetyltransferase gene (bar) was used as a selectable marker. pSUBG, a control vector containing the bar cassette only; pSUBGX4S, a sense construct containing the coding region of OsEXP4; pSUBGX4As, an antisense construct containing the coding region of OsEXP4 in the reverse direction, nos, nopaline synthase.</td>
<td>(A) NTR, CON, S-22, S-23, S-24, S-3, AS-1, AS-2, AS-3, AS-4, AS-5;</td>
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<tr>
<td>(B) OsEXP4 constructs using the In2-2 promoter. The hygromycin phosphotransferase gene (hpt) was used as a selectable marker. pKC101, a control construct containing the In2-2 promoter and the hpt cassette; pKCGUS, a control construct containing the β-glucuronidase gene (GUS) fused to the In2-2 promoter; pKCX4S, a sense construct containing the coding sequence of OsEXP4 fused to the In2-2 promoter; pKCX4As1, an antisense construct containing the coding sequence of OsEXP4 fused to the In2-2 promoter in the reverse direction; pKCX4As2, an antisense construct containing an OsEXP4-specific sequence (3’ untranslated region [UTR]) fused to the In2-2 promoter in the reverse direction. CaMV, Cauliflower mosaic virus.</td>
<td>Relative signal density (%)</td>
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<td><strong>A</strong> 4.0 kb</td>
<td><strong>B</strong> Transgenic lines</td>
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<td><strong>B</strong> Relative signal density (%)</td>
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Five micrograms of DNA was digested with BamHI and HindIII, separated on a 1% agarose gel, and subjected to DNA gel blot analysis. A purified PCR product from the 3’ untranslated region of OsEXP4 was radiolabeled with 32P-dCTP by random priming and used as a probe.

(A) The arrow at 1.1 kb indicates the position of the OsEXP4 transgene; the arrow at 4.0 kb indicates the position of the endogenous OsEXP4 gene. AS, antisense gene; CON, control transgenic plant carrying the pSUBG vector; NTR, nontransformed plant; S, sense gene.

(B) Relative signal density was calculated to estimate the copy number of transgenes. The ratio between the signal intensity of the transgene and the signal intensity of the endogenous gene is shown as a percentage.
concentration of 2-CBSU by germinating seeds from transgenic plants, which carried the pKCGUS construct (Figure 1B), on wet filter paper or under submergence. For germination on wet filter paper and growth in air, 50 mg/L 2-CBSU was the optimal concentration that induced expression of the β-glucuronidase gene without retarding the growth of the coleoptile and mesocotyl; under submergence, 5 mg/L 2-CBSU was the optimal concentration (data not shown). When T₁ seeds of OsEXP4 sense and antisense transgenic plants were germinated on wet filter paper and seedlings were grown in air in the presence of 2-CBSU, significant changes in coleoptile and mesocotyl length were found. In sense transgenic seedlings, the length of coleoptiles and mesocotyls increased by up to 31 and 97%, respectively, whereas the coleoptile and mesocotyl length of antisense seedlings decreased by up to 28 and 43%, respectively (Figures 6B to 6E, Table 1).

Immunoblot analysis with polyclonal antiserum raised against OsEXP4 indicated that the level of OsEXP4 increased in sense transgenic seedlings and decreased in antisense seedlings upon treatment with 2-CBSU (Figures 6B to 6E). Seedlings har-

Figure 3. Expression of the OsEXP4 Transgene in Mature Leaves of Adult Transgenic Plants. Twenty micrograms of RNA from each transgenic line was separated on 1% formaldehyde agarose gels and subjected to RNA gel blot analysis. (A) RNA gel blot analysis of sense (S) transgenic plants. A radiolabeled antisense OsEXP4 RNA from the 3’ untranslated region of OsEXP4 cDNA was used as a probe. CON, control transgenic plant. (B) RNA gel blot analysis of antisense (AS) transgenic plants. A radiolabeled sense RNA from the 3’ untranslated region of OsEXP4 cDNA was used as a probe.

Figure 4. Morphology of Adult Transgenic Plants Carrying Constructs with Constitutive OsEXP4 Sense and Antisense Expression. (A) Shown are the antisense transgenic plant AS-4 (a), the control plant C6-23 harboring the pSUBG vector (b), the tall sense transgenic plant S-4 (c), and the short sense transgenic plant S-24 with bush-like leaves (d). (B) Sense transgenic plants exhibiting abnormal morphologies. Shown are plant S-21 with multiple leaves at the same nodes (e) and plant S-26 with an adventitious secondary branch on the main stem (f). The white arrow (magnified in the inset) indicates an adventitious branch on the main tiller of sense plant S-26.
boring an OsEXP4-specific antisense construct (Figure 6E) showed the same reduction in growth as did seedlings expressing the full-length antisense construct (Figure 6D), even though the OsEXP4 protein content was lower in seedlings with the specific antisense construct than in seedlings carrying the full-length antisense transgene. In control transgenic seedlings, coleoptile and mesocotyl growth was not affected by treatment with 2-CBSU, although root growth was inhibited, and no change in the level of expansin protein was observed (Figure 6A). These results demonstrate that altering the level of OsEXP4 influenced seedling growth and that the mesocotyl was more sensitive to changes in OsEXP4 content than was the coleoptile. This trend was consistent in all independent transgenic lines (Table 1).

The coleoptiles of submerged T1 sense and antisense transgenic seedlings grew to more than twice the length of air-grown coleoptiles (cf. Figures 6A and 7A). Expression of OsEXP4 induced by treatment with 2-CBSU did not promote additional coleoptile growth (Figure 7B). However, coleoptile growth in 2-CBSU–treated antisense seedlings was reduced by 25% (Figures 7C and 7D). Growth of the mesocotyl was inhibited severely in submerged seedlings in the presence and absence of 2-CBSU (Figures 7A to 7D).

Expression of OsEXP4 Affects the Enlargement of Mesocotyl Cells

To understand the cellular basis for expansin-mediated growth, we measured cell length and width in mesocotyls harboring sense and antisense constructs of OsEXP4. Mesocotyl cells adjacent to the coleoptilar node are meristematic and contribute to growth by adding new cells to the mesocotyl (Watanabe et al., 2001). Cells in the center of the mesocotyl are the longest among the cells that constitute the mesocotyl, and they have relatively even sizes. Twenty cells were chosen from the center of mesocotyls, and the average cell size was determined. In the presence of 2-CBSU, the average cell length increased by up to 58% in mesocotyls of lines overexpressing OsEXP4, whereas the average cell length in the antisense transgenic line showed a 22% reduction (Figures 8B to 8D). Under the same conditions, cell width was not affected.
Expression of OsEXP4 Affects Cell Wall Extensibility

To correlate OsEXP4 activity and seedling growth, we examined the acid-induced extensibility of cell walls isolated from air-grown coleoptiles that had been treated with or without 2-CBSU. For these experiments, we used 1-cm sections from the apical region of coleoptiles because this is the zone with the highest growth rate and cell wall extensibility (Cho and Kende, 1997c). Cell wall extensibility of coleoptiles from sense transgenic lines increased by up to 32%, whereas that of antisense transgenic lines decreased by up to 20% (Figures 9B to 9E). The extensibility of cell walls from submerged seedlings could not be examined because their coleoptiles were very fragile and did not withstand a load of even 1 g.

DISCUSSION

α-Expansins Are Highly Redundant in Rice and May Not Be the Only Cell Wall–Loosening Factors

One way to determine the role of expansins in plant growth and development is to increase or decrease endogenous expansin content by transgenic methods. The success of this approach depends in part on the redundancy of expansins in a given plant or organ. Thanks to the availability of the nearly complete genome sequences of japonica (Goff et al., 2002) and indica rice (Yu et al., 2002), we know that the rice genome contains at least 33 α-expansin and 18 β-expansin genes (Y. Lee and H. Kende, unpublished data). We have studied the expression of 26 α-expansin and 14 β-expansin genes in various organs of rice and found a considerable overlap in their expression patterns (Cho and Kende, 1997c; Lee and Kende, 2001, 2002). In coleoptiles, for example, at least six α-expansin and four β-expansin genes are expressed (Lee and Kende, 2002). Because of this redundancy, one may not observe dramatic changes in growth by altering the expression of any one expansin gene, even if it encodes a major expansin protein, such as OsEXP4.

In addition to the expansins, hydroxyl radicals also induce cell wall loosening and growth (Schopfer, 2001; Schopfer et al., 2002). It would not be surprising if a complex physiological process, such as extension of the plant cell wall, were regulated by a number of factors that act in concert or in response to specific stimuli.

Constitutive Overexpression of OsEXP4 Yielded Pleiotropic Phenotypes in Adult Rice Plants

Overexpression of OsEXP4 in the sense and antisense orientations under the control of the constitutive Ubi-1 promoter yielded pleiotropic phenotypes in adult rice plants (Figures 4A, 4B, and 5A to 5C). The promotion of growth in a subpopulation of sense transformants was attributable to enhanced internodal elongation and is consistent with the observation that GA- and submergence-induced growth in deepwater rice is preceded by increased OsEXP4 transcript accumulation (Cho and Kende, 1997c). The inhibition of growth in the majority of sense transformants was not caused by gene silencing, because these transgenic plants produced even higher levels of OsEXP4 transcripts than did sense transformants whose growth was enhanced (Figure 3A). We propose two possible explanations for the stunted growth of OsEXP4 overexpressors. Inhibition of growth could be caused by morphological changes at the shoot apical meristem, specifically by the formation of additional leaves. It has been shown by in situ hybridization in rice and tomato that expansin genes are highly expressed in cells of...
the shoot apical meristem that give rise to leaf primordia (Cho and Kende, 1998; Reinhardt et al., 1998).

The role of expansins in leaf formation has been demonstrated even more directly by Fleming et al. (1997), who elicited the development of leaf-like structures at the shoot apical meristem of tomato plants by local application of expansin protein, and by Pien et al. (2001), who induced local expression of an expansin gene at the shoot apical meristem, leading to the formation of an entire leaf. Therefore, it is likely that ectopic, constitutive expression of OsEXP4 disturbs the organization of the shoot apical meristem and that internodal growth is restricted by the formation of additional leaves or even tillers (Figures 4B and 5B). Alternatively, it is conceivable that the formation of different organs depends on different threshold levels of expansin, with lower levels promoting stem elongation and higher levels promoting leaf development. Changes in the morphological organization of the shoot apical meristem also may result in the observed delay of flowering in sense transformants (Figure 5C). Because transgenic plants that overexpress sense OsEXP4 were sterile, no experiments beyond the T<sub>0</sub> generation could be performed with them. For this reason, we initiated a new series of rice transformations using expansin constructs under the control of the inducible In2-2 promoter.

Antisense expression of OsEXP4 did not result in any striking novel phenotypes. The distribution of antisense plants was shifted toward sizes shorter than those of control plants, the number of leaves was unchanged, and the flowering time was advanced (Figures 4A and 5A to 5C). The latter effect can be attributed either to decreased interference by additional leaf primordia at the shoot apical meristem or to the development of floral structures at a lower expansin threshold. We propose two explanations for the relatively minor effect of antisense OsEXP4 expression on the growth and development of adult rice plants. First, the expression of the antisense constructs was low in all transgenic lines (Figure 3B). Second, because eight α-expansin and five β-expansin genes are expressed in rice internodes (Lee and Kende, 2002), reducing the expression of one expansin gene may have only subtle effects.

### Table 1. Effect of Expansin Levels on the Growth of Transgenic Rice Seedlings

<table>
<thead>
<tr>
<th>Germination and Growth Conditions</th>
<th>Transgenic Lines</th>
<th>Coleoptile Length (% of Control)</th>
<th>Mesocotyl Length (% of Control)</th>
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<tbody>
<tr>
<td>Air-grown Sense lines</td>
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<td></td>
<td></td>
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<tr>
<td>S1</td>
<td>113</td>
<td>135</td>
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<td>S2</td>
<td>115</td>
<td>137</td>
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<td>S3</td>
<td>119</td>
<td>150</td>
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<tr>
<td>S4</td>
<td>124</td>
<td>158</td>
<td></td>
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<tr>
<td>S5</td>
<td>121</td>
<td>197</td>
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<tr>
<td>S6</td>
<td>123</td>
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<tr>
<td>S7</td>
<td>127</td>
<td>155</td>
<td></td>
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<tr>
<td>Antisense lines</td>
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<td></td>
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</tr>
<tr>
<td>As1-1</td>
<td>79</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>As1-2</td>
<td>77</td>
<td>59</td>
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<td>As1-3</td>
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<tr>
<td>As2-4</td>
<td>86</td>
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<td>As2-5</td>
<td>76</td>
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<td></td>
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<tr>
<td>As2-6</td>
<td>79</td>
<td>64</td>
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<tr>
<td>Submergence Antisense lines</td>
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<tr>
<td>As1-1</td>
<td>76</td>
<td>N.D.&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>As1-2</td>
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<tr>
<td>As2-6</td>
<td>65</td>
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Transgenic seeds were germinated on wet filter paper with or without 50 mg/L 2-CBSU (air-grown plants) or submerged with or without 5 mg/L 2-CBSU. Coleoptile and mesocotyl lengths were measured at 3 to 5 days after the start of imbibition and growth at 30°C in darkness. Each transgenic line represents an independent transformation event.

<sup>a</sup>N.D., not determined.

### Induced Expression of OsEXP4 Affected the Growth of Both the Coleoptile and the Mesocotyl

To investigate the function of OsEXP4 in specific organs and to solve the sterility problem encountered in plants with constitutive overexpression of OsEXP4, we transformed rice with sense and antisense constructs of OsEXP4 under the control of the inducible In2-2 promoter. In air-grown seedlings, overexpression of OsEXP4 increased the level of the OsEXP4 protein and promoted the growth of the coleoptile and the mesocotyl. The relative increase in the growth of the mesocotyl was greater than that of the coleoptile (Figures 6B and 6C, Table 1). Expression of antisense OsEXP4 constructs reduced the elongation of the coleoptile and, to a greater extent, of the mesocotyl (Fig-
The difference in responsiveness to increased and decreased expansin content in the coleoptile and mesocotyl may reflect the redundancy of expansins in these two organs or the susceptibility of the respective cell walls to expansin action.

Submergence and reduced partial pressures of oxygen enhance the growth of rice coleoptiles, and this promotion of growth is correlated with increased expression of the expansin genes OsEXP2 and OsEXP4 (Huang et al., 2000). Whereas elongation of the coleoptile was stimulated approximately two-fold by submergence, growth of the mesocotyl was inhibited severely (cf. Figures 6 and 7). Increasing the level of OsEXP4 protein by treatment with 2-CBSU did not enhance the elongation of either organ (Figure 7B), presumably because the submerged coleoptiles already were growing at maximum rate and because the growth of the mesocotyl requires oxygen concentrations that are higher than those in nonaerated water. However, reducing the level of OsEXP4 by the expression of OsEXP4 antisense constructs decreased the growth of submerged coleoptiles (Figures 7C and 7D).

Figure 7. Growth of Submerged Transgenic Seedlings in the Presence and Absence of the Inducer 2-CBSU.

(A) Growth of control seedlings carrying the pKC vector (pKC1-1).
(B) Growth of OsEXP4 sense transgenic seedlings (pKCX4S1).
(C) Growth of OsEXP4 antisense transgenic seedlings (pKCX4As1-3).
(D) Growth of OsEXP4 antisense transgenic seedlings (pKCX4As2-5).

Seeds were submerged and germinated in 20-mL scintillation vials containing 15 mL of distilled water (–2-CBSU) or 5 mg/L 2-CBSU solution (+2-CBSU). Coleoptile and mesocotyl lengths were measured after 3 days of growth under submerged conditions. Immunoblot analysis of the coleoptile and mesocotyls was performed with polyclonal antiserum raised against the OsEXP4–glutathione S-transferase fusion protein. The error bars represent standard errors (n = 12 to 21).

Figure 8. Measurement of Cell Sizes in the Mesocotyls of Transgenic Seedlings.

(A) Cell length and width in the mesocotyls of control transgenic seedlings carrying the pKC vector (pKC1-1).
(B) Cell length and width in the mesocotyls of OsEXP4 sense transgenic seedlings (pKCX4S1).
(C) Cell length and width in the mesocotyls of OsEXP4 sense transgenic seedlings (pKCX4S5).
(D) Cell length and width in the mesocotyls of OsEXP4 antisense transgenic seedlings (pKCX4As2-5).

Mesocotyls of transgenic seedlings were excised after germination and growth of seedlings for 3 days with (+) or without (–) 50 mg/L 2-CBSU, embedded in Paraplast Plus, sectioned, and stained with 0.1% toluidine blue. Cell length and width were measured with a microscope using an ocular micrometer. The error bars represent standard errors (n = 15).
Measurements of Cell Size and Cell Wall Extensibility
Support the Model of Expansin Action

The model of expansin action postulates that expansins disrupt noncovalent bonds between cellulose microfibrils and matrix polysaccharides or between matrix polysaccharides themselves, thereby permitting loosening of the cell walls and turgor-driven growth of the cell (reviewed by Cosgrove, 2000). If this model is correct, the increased and decreased growth of rice seedlings containing higher or lower levels of OsEXP4 proteins, respectively, should be reflected in cell sizes and cell wall extensibility. Our experiments confirmed this prediction.

The small size of the mesocotyl facilitated the measurement of cell sizes from exactly the same central region of each mesocotyl. This procedure reduced variability and made comparisons between treatments more reliable. There was a direct relationship between the size of the mesocotyl, the level of OsEXP4, and the length of mesocotyl cells (Figures 6 and 8).

Cell wall extensibility in the apical, growing region of coleoptiles was more than twofold greater than in the basal, nongrowing region and was correlated with the level of endogenous OsEXP4, and the length of mesocotyl cells (Figures 6 and 8).

Cell wall extensibility in coleoptiles from transgenic seedlings carrying the pKC vector (pKC1-1).
(B) Cell wall extensibility in coleoptiles of OsEXP4 sense transgenic seedlings (pKCX4S1).
(C) Cell wall extensibility in coleoptiles of OsEXP4 sense transgenic seedlings (pKCX4S5).
(D) Cell wall extensibility in coleoptiles of OsEXP4 antisense transgenic seedlings (pKCX4As1-3).
(E) Change in cell wall extensibility in coleoptiles of OsEXP4 antisense transgenic seedlings (pKCX4As2-5).

Acid-induced cell wall extension of coleoptile sections was measured after germination and growth of seedlings for 3 days with (+) or without (−) 50 mg/L 2-CBSU treatment. One-centimeter sections were excised from the distal end of the coleoptiles, frozen and thawed, and used for measurements of extensibility with an extensometer. The values shown represent the average cell wall extension of coleoptile sections at 30 min after the start of incubation in acetate buffer, pH 4.5. The error bars represent standard errors (n = 15 to 20).

Conclusions

Experiments with transgenic plants expressing expansin genes at either increased or decreased levels gave mixed results with respect to the validity of the expansin-growth hypothesis. Overexpression of an expansin gene in tomato plants reduced the size of the fruits (Brumme et al., 1999), stunted the growth of the shoot, and inhibited cell elongation (Rochange et al., 2001). Null mutations in four expansin genes did not alter the phenotype of the moss P. patens. Therefore, the results of these experiments did not support the expansin-growth hypothesis. However, the development and growth of leaves were affected by induced and reduced expression of expansin genes in a manner consistent with the expansin hypothesis (Cho and Cosgrove, 2000; Pien et al., 2001). In spite of the great redundancy of both α- and β-expansins in rice and the possibility that other wall-loosening agents also may mediate growth, our results clearly support the hypothesis that expansins have cell wall-loosening and growth-promoting functions in general and that endogenous α-expansins are active in rice even though applied α-expansin preparations elicited little wall loosening in isolated cell walls of maize, barley, and oat coleoptiles (McQueen-Mason et al., 1992; Li et al., 1993). Because a β-expansin, the Zea m1 pollen allergen, showed high cell wall-loosening activity in maize cell walls (Cosgrove et al., 1997), it will be interesting to assess the effect of manipulating the level of endogenous β-expansin alone and in combination with an α-expansin on cell wall properties and growth in rice and other grasses.

METHODS

Construction of the Binary Vectors pSUBG and pKC101

For constitutive expression of transgenes, we constructed the superbinary vector pSUBG by ligating the Ubi-1:BAR cassette from pAHC25 (Christensen and Quail, 1996) to the binary vector pSB11 (Komari et al., 1996). For induced expression of transgenes, a binary vector, pKC101, was constructed by replacing the Ubi-1 promoter in the binary vector pGA1611 (Kang et al., 1998) with the In2-2 promoter from the binary vector pTDS136 plasmid (Veylder et al., 1997). The resulting constructs were introduced into Agrobacterium tumefaciens strain LBA4404.

Cloning of OsEXP4 cDNA in Binary Vectors and Agrobacterium Transformation

For insertion into the appropriate vectors, we prepared a full-length OsEXP4 construct by amplifying OsEXP4 cDNA by PCR using primers.
that included at both ends a BamHI recognition site (5'-GCCAGGCTT-TAGACAGGATCCCA-3') and 5'-ATTCCGTGGATCCCATCCATCCTC-3'; the BamHI site is underlined. The PCR products were inserted at the BamHI site into the pSUBG vector. Sense (pSUBGX4A5) and antisense (pSUBGX4A3) orientations were confirmed by PCR with the forward primer 5'-TCTGGATATCATGGATGAT-3' from the Ubi-1 promoter and the reverse primers for the sense orientation, 5'-GCCGTGCGCA-AGAAGAGGAGAAGAGA-3'; for the antisense orientation, 5'-GCC-TTCTCTTTATAGTGAGAAGGT-3' from inside the OsEXP4 cDNA. Confirmed constructs were introduced into Agrobacterium LBA4404 (pSB1 with the tetracycline resistance gene), and colonies with recombinant plasmids were selected on solidified AB medium (Chilton et al., 1974) containing 30 mg/L spectinomycin and 5 mg/L tetracycline.

For inducible promoter constructs, various parts of OsEXP4 were amplified and inserted into the pKC101 vector. As a control, the β-glucuronidase gene (GUS) was amplified by PCR with the primers 5'-GTGGAT-CGCCCTCTATTACCT-3' and 5'-CGAAGCTTCTGGATCCGCTCTC-3' from inside the pTD3136 plasmid containing the GUS coding sequence and inserted into the pKC101 vector (pKCGUS). For overexpressor and full-length antisense constructs, intact OsEXP4 was amplified by PCR with primers containing a HindIII recognition site at both ends (5'-CCCAAGC-TCCGACACATCCACC-3' and 5'-TCAAGCTTCTGGATCCGATAAAT-3'; the HindIII site is underlined) and inserted into the pKC101 vector. The sense (pKX4A5S) and antisense (pKX4A5A) constructs were sequenced and used for transformation of rice. The 3' untranslated region of OsEXP4 was amplified by PCR with primers that included the SacI recognition site at both ends (5'-TGGACGCTGCAGCGCAGACA-3' and 5'-TAGAAGCTGCGCTGGTTT-3'; the SacI site is underlined) and was inserted into the pKC101 vector in the antisense orientation (pKX4As). All constructs were introduced into Agrobacterium LBA4404 cells by the freeze-thaw method (An et al., 1988). Transformed Agrobacterium cells were screened on solidified AB medium containing the appropriate antibiotics after incubation for 3 days at 28°C in darkness. Colonies were picked and cultured in 2 mL of liquid AB medium overnight at 28°C; for the antisense orientation, 5°C.

Rice seeds (Oryza sativa cv Taipei 309) were obtained from Thomas Hodges (Purdue University, West Lafayette, IN). The plants were grown in a growth chamber according to Stünzi and Kende (1989). Embryogenic calli were derived from mature seeds on MSS2D medium (Kant et al., 2001) containing MS salts (Murashige and Skoog, 1962), N6 vitamins (Chui et al., 1975), 30 g/L sucrose, and 2 mg/L 2,4-D. The calli were maintained at 28°C on MS2D medium (Kant et al., 2001) containing 30 g/L maltose instead of sucrose. Agrobacterium LBA4404 carrying constructs with the constitutive Ubi-1 promoter (pSUBG) was grown in 20% maltose instead of sucrose. Agrobacterium LBA4404 carrying constructs with the inducible In2-2 promoter (pKC) was grown under the same conditions with 300 mg/L acetosyringone, and 5 mg/L tetracycline overnight at 28°C and 300 rpm. Agrobacterium LBA4404 carrying constructs with the inducible In2-2 promoter (pKC) was grown under the same conditions with 300 mg/L hygromycin instead of spectinomycin.

The next day, cells were harvested by centrifugation at 5000g for 10 min at 4°C, resuspended in PIM2 medium (Kant et al., 2001) containing 1% glucose, 75 mM Mes, pH 5.6, 2 mM sodium phosphate buffer, pH 5.6, 1x AB salts (Chilton et al., 1974), and 200 μM acetosyringone, and cultured at 28°C for 16 h with vigorous shaking at 300 rpm. These agrobacteria were cocultivated on 2N6-AS medium (Hei et al., 1994) containing 100 μM FeEDTA, 100 μM acetosyringone, and 3 g/L Gelrite gellan gum (Sigma) at 22°C in darkness for 3 days with embryogenic calli that had been precultured on MSM2D medium for 3 days. The cocultivated calli were washed with sterile distilled water containing 100 mg/L cefotaxime (Sigma) and incubated in darkness at 28°C for 3 to 4 weeks on N6CS medium (Aldemita and Hodges, 1996) containing N6 salts, N6 vitamins, 30 g/L sucrose, 100 mg/L cefotaxime, 250 mg/L carbenicillin (Sigma), and appropriate selectable antibiotics such as 40 mg/L hygromycin (Calbiochem) or 8 to 10 mg/L phosphinothricin (Sigma).

DNA and RNA Gel Blot Analysis of Adult Transgenic Plants

Genomic DNA was isolated from mature leaves of T0 transgenic plants using the cetyltrimethylammonium bromide method (Rogers and Bendich, 1988). Genomic DNA (5 μg) was digested with BamHI and HindIII overnight, separated on a 0.8% agarose gel, and blotted onto a Hybond-N membrane (Amersham Pharmacia). Blots were hybridized to a probe consisting of 100 ng of a 32P-labeled OsEXP4-specific DNA fragment amplified by PCR with primers corresponding to the 3' untranslated region of OsEXP4 (5'-CCAGGCTTCCGACACATCCACC-3' and 5'-ATTCCGTTGAAACGGCGCATCCCTC-3'). Hybridization was performed by using non-radioactive nucleotide probes. Membranes were exposed to autoradiography film (Amersham Pharmacia). The density of each signal on the autoradiography film was determined by ImageQuant analysis (Molecular Dynamics, Sunnyvale, CA), and the approximate copy number of transgenes was calculated as follows: density of transgene signal/density of endogenous OsEXP4 signal.

Total RNA was isolated from mature leaves using the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987), separated on a 1% formaldehyde agarose gel (20 μg/lane) (Ausbel et al., 1987), and blotted onto a Hybond-N+ membrane. The RNA was hybridized to a probe consisting of 50 ng of a 32P-UTP–labeled sense riboprobe from the 3' untranslated region of OsEXP4 cDNA for antisense transgenic lines or with a 32P-UTP–labeled antisense riboprobe from the 3' untranslated region of OsEXP4 cDNA for sense transgenic lines. Riboprobes were prepared from a plasmid containing the EagI-HindIII fragment of OsEXP4 cDNA (Cho and Kende, 1998) by in vitro transcription using the T3/T7 transcription kit (Boehringer Mannheim).

Preparation of Polyclonal Antibody against OsEXP4

The DNA sequence corresponding to the C-terminal region of OsEXP4 (Val-95 to Phe-246) was amplified using the primers 5'-TGATCGCTGGAGCCACCACCC-3' and 5'-GTTAAAAACGAGGCAGACTG-3'. The amplified fragment of OsEXP4 cDNA was digested with BamHI and XhoI restriction enzymes, inserted into the pGEX-4T-1 expression vector, and cloned in Escherichia coli DH5α, and the sequence of the inserted DNA was...
confirmed. The cloned OsEXP4 cDNA was introduced into the expression host E. coli BL21 (DE3). Induction and purification of the cloned protein were performed according to the manufacturer’s protocol (Amer- sham Pharmacia).

Antisera against the OsEXP4 fragment were raised in female New Zealand White rabbits at Cocalico Biologicals (Reamstown, PA). After an initial injection of 100 μg of SDS-PAGE-purified OsEXP4 protein in Freund’s complete adjuvant, the animals were boosted three times with 100 μg of antigen in incomplete Freund’s adjuvant at 4-week intervals.

Immunoblot Analysis of Expansin from Transgenic Seedlings

Cell wall protein was extracted from coleoptiles and mesocotyls as described previously (Cho and Kende, 1997a) but on a smaller scale and with minor modifications. The tissue was homogenized in a mortar in cell wall protein extraction buffer consisting of 20 mM Hepes/NaOH, pH 7.0, 2 mM metabisulfite, 2 mM EDTA, and 1 M NaCl. The homogenate was transferred to 1.5-mL Eppendorf tubes and incubated for 1 h at 4°C. After incubation, the homogenate was centrifuged at 15,800g for 10 min at 4°C, and the supernatant was used as a cell wall protein sample. Immuno blot analyses were performed according to a standard protocol. Protein samples were separated on 12% polyacrylamide gels and electroblotted onto a nitrocellulose membrane (Hybond-C; Amersham Pharma- cia) in a transfer buffer containing 15.6 mM Tris and 120 mM Gly for 1 h at 100 V. The membrane was blocked with 3% BSA in TBST buffer (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) for 1 h, followed by incubation with OsEXP4 rabbit polyclonal antiserum (diluted 1:10,000) for 1 h in TBST buffer containing 0.5% BSA.

After three rinses in TBST for 15 min each, the membrane was incu- bated in goat anti-rabbit IgG-conjugated horseradish peroxidase (diluted 1:10,000) for 1 h in TBST buffer containing 0.5% BSA. After three rinses in TBST for 30 min each, OsEXP4 was visualized on the immuno blot with the enhanced chemiluminescence system (Amersham Pharma- cia) according to the manufacturer’s protocol. Whereas the antibody against OsEXP4 detects only a single, sharp protein band of 24.5 kD, corresponding to the molecular mass of OsEXP4 on an immunoblot (data not shown), it is likely that it cross-reacts to some extent with other α-expansins such as OsEXP2, whose molecular mass is 24.9 kD and whose amino acid sequence is similar to that of OsEXP4.

Measurement of Morphological and Physiological Characteristics of Adult Transgenic Plants Carrying Constitutive pSUBG Constructs

The height of adult plants was determined by measuring the length of the main tiller of each transgenic plant from the crown of the roots to the flag leaf. To check the effect of OsEXP4 expression on leaf formation, the number of leaves on the main tiller was counted. The time between transplanting of regenerant plantlets into soil and flower emergence was recorded as the flowering time.

Measurement of Seedling Growth and Mesocotyl Cell Size in Transgenic Seedlings Carrying Inducible Gene Constructs

Seeds were sterilized in 1.2% sodium hypochlorite for 20 min, washed three times in sterile distilled water for 20 min with vigorous shaking, and germinated on filter paper soaked with distilled water or with 50 mg/L N-(aminocarbonyl)-2-chlorobenzenesulfonamide dissolved in distilled water. To screen for transgenic seeds, 50 mg/L hygromycin was added to the germination solution. After germination for 3 days at 30°C in darkness, coleoptile and mesocotyl lengths were measured. For cell size measurements, mesocotyls were excised, fixed in formalin-acetic acid-alcohol fixative, dehydrated by passage through increasing concentrations of ethyl alcohol, cleared in xylene, and embedded in Paraplast Plus medium (Oxford Labware, St. Louis, MO). Mesocotyls in Paraplast Plus blocks were sectioned to 10 μm thickness, and the mesocotyl sections were stained with 0.1% toluidine blue and examined with a light microscope.

We determined, in the central region of the mesocotyl, the total length of 20 cells in the third cell file from the epidermis on both sides of the section using a microscope with an ocular micrometer and calculated the average cell length. Cell width was measured in the same cells at the same time, and the average cell width was calculated. Fifteen sections from each transgenic line were examined. To measure the growth of submerged seedlings, seeds were germinated and seedlings were grown in darkness in 20-mL scintillation vials containing 15 mL of distilled water with or without 5 mg/L N-(aminocarbonyl)-2-chlorobenzenesulfonamide. To screen for transgenic seeds, 10 mg/L hygromycin was added to the germination solution. The insertion of transgene constructs was confirmed in all transgenic lines by PCR with genomic DNA from primary leaves as a template.

Measurement of Cell Wall Extensibility in Coleoptiles of Transgenic Seedlings

Cell wall extensibility was measured according to the method of Cosgrove (1989), with a modification described previously (Cho and Kende, 1997a). Coleoptile sections, 10 mm long including the tip, were excised, frozen at −80°C, abraded with 300-mesh carbonabondum slurry (Fisher Scientific), thawed, and pressed between filter paper to remove water and cell sap. Each segment was inserted between two clamps 5 mm apart, placed into an extensometer equipped with an angular displacement transducer (Kutschera and Briggs, 1987), and subjected to con- stant tension using a weight of 10 g. The segments were submerged in 50 mM Heps-Tris buffer, pH 6.8, for 20 min, after which the buffer was replaced with 50 mM sodium acetate buffer, pH 4.5. The increase in section length was traced with a chart recorder, and the extension after 30 min of incubation at pH 4.5 was plotted (Figure 9).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Number

The accession number for OsEXP4 is U85246.

ACKNOWLEDGMENTS

We thank Thomas Hodges (Purdue University) for rice seeds, for teach- ing us the procedures of rice transformation, and for helpful advice; Howard Hershey (DuPont Company, Wilmington, DE) for the generous gift of the pTDS 136 plasmid containing the In2-2 promoter and 2-CBSU and for helpful suggestions; Yukoh Hiei (Japan Tobacco, Iwata, Japan) for providing Agrobacterium strain LBA4404 and the plasmid pSB11; Peter Quail (University of California, Berkeley) for the gift of the pAHC25 plasmid; and Gyungheun An (Pohang University of Science and Technol- ogy, Pohang, Republic of Korea) for the gift of the pGA1611 binary vector and helpful suggestions concerning rice transformation. This re- search was supported by Grant IBN 0076524 from the National Science Foundation and Grant DE-FG-02-91ER20021 from the U.S. Department of Energy.

Received March 14, 2003; accepted March 24, 2003.

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Regulation of Expansin Gene Expression Affects Growth and Development in Transgenic Rice Plants
Dongsu Choi, Yi Lee, Hyung-Taeg Cho and Hans Kende
Plant Cell 2003;15;1386-1398; originally published online May 8, 2003;
DOI 10.1105/tpc.011965

This information is current as of June 11, 2017

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