Role of Auxin-Induced Reactive Oxygen Species in Root Gravitropism[1]

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We report our studies on root gravitropism indicating that reactive oxygen species (ROS) may function as a downstream component in auxin-mediated signal transduction. A transient increase in the intracellular concentration of ROS in the convex endodermis resulted from either gravistimulation or unilateral application of auxin to vertical roots. Root bending was also brought about by unilateral application of ROS to vertical roots pretreated with the auxin transport inhibitor *N*-1-naphthylphthalamic acid. Furthermore, the scavenging of ROS by antioxidants (*N*-acetylcysteine, ascorbic acid, and Trolox) inhibited root gravitropism. These results indicate that the generation of ROS plays a role in root gravitropism.

Since Cholodny (1926) and Went (1926) discovered that directional auxin transport occurs upon gravistimulation, the mechanism of auxin transport is well established. According to the mechanism, the gravitropic stimulation induces asymmetric auxin movement, and the localized auxin in turn causes gravitropic curvature (Young et al., 1990; Dolan, 1998; Rosen et al., 1999). These results indicate that auxin is indeed essential for gravitropism. Several lines of evidence suggest that the second messengers, Ca^{2+} and inositol 1,4,5-triphosphate (IP_3), are involved in root gravitropism (Lee et al., 1983; Perera et al., 1999). However, the relationship between auxin and second messengers is still unknown.

Although reactive oxygen species (ROS) such as superoxide anions and H_2O_2 are generally considered to be toxic byproducts of respiration, recent evidence suggests that the production of ROS might be an integral component of intracellular signaling (Krieger-Brauer and Kather, 1992; Finkel, 1998; Rhee et al., 2000). In mammalian cells, a variety of extracellular stimuli have been shown to induce a transient increase in the intracellular concentration of ROS, and specific inhibition of the ROS generation results in a complete blockage of stimulus-dependent signaling (Sundaresan et al., 1995; Bae et al., 1997). Also, several lines of evidence suggest that ROS serve as signaling molecules in plants. It has been shown that ROS mediate systemic signal networks for plant defense (Chen et al., 1993; Greenberg, 1996; Pennell and Lamb, 1997; Alvarez et al., 1998). ROS stimulate cell wall stiffening and apoptosis in infectious regions, preventing the transmission of infectious particles (Lamb and Dixon, 1997; Delledone et al., 1998; Potikha et al., 1999; Grant and Loake, 2000). Recent evidence suggests that the plant hormone abscisic acid-mediated H_2O_2 generation and the H_2O_2-activated Ca^{2+} are important in stomatal closing (Pei et al., 2000). However, the potential of ROS as a second messenger in root gravitropism is still unclear. In this report, we reveal the role of ROS in root gravitropism.

RESULTS AND DISCUSSION

Gravity Induces Asymmetric ROS Generation

To verify the role of ROS in plant gravitropism, the generation of ROS in maize (*Zea mays*) primary root was investigated following gravistimulation by placing the root horizontally. Upon gravistimulation of maize primary root, the intracellular concentration of ROS, as measured by the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}), increased by 3-fold within 1 h and then declined to the basal level (Fig. 1A). The portion of the root we refer to as zone 1 is the apical 4 mm of the root and includes the root cap, meristem, and the apical half of the root elongation zone. Zone 2 extends from 4 to 8 mm from the root tip and includes the basal half of the elongation zone. ROS appeared in zone 1 of primary roots in response to gravity, whereas there was little change in ROS in zone 2. Longer gravistimulation of the root also led to the accumulation of ROS in zone 2. As ROS are very diffusible molecules, it is likely that the accumulation of ROS in zone 2 results from diffusion of ROS into zone 2 from the zone 1. Salicylic acid is a well-known inducer of ROS production in plants (Chen et al., 1993). However, no difference of ROS generation in salicylic acid-treated root was detectable between the zone 1 and zone 2. It is likely that the generation of ROS following gravistimulation is more specific to zone 1 than to zone 2.

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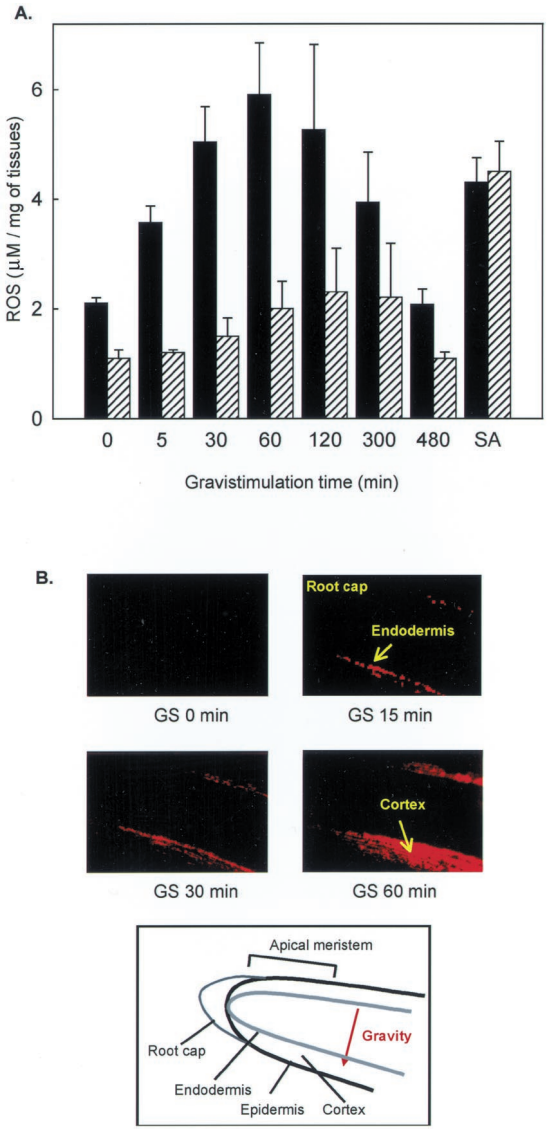

Figure 1. Generation of ROS during gravitropism in maize roots. A, Time course of gravistimulation-induced ROS generation. The roots were oriented horizontally for the indicated time and then cut into two parts. Zone 1 (black bars) contains the apical end of root to 0.4 cm, and zone 2 (hatched bars) contains 0.4 to 0.8 cm from root tip. SA, Salicylic acid-treated maize roots. The segments were subjected to ROS measurement assay (OXIS, Portland, OR). Values are the means ± SE for five independent experiments. B, Asymmetric generation of ROS by gravistimulation. Gravistimulated or control roots were dissected and stained with a 0.003% (w/v) dihydrorhodamine-123 solution for 10 min. Fluorescence intensity of oxidized rhodamine was observed with a fluorescence microscope (Zeiss, Jena, Germany; excitation = 485 nm, emission = 535 nm). Experiments were repeated at least five times with similar results.

Gravistimulation-induced ROS were monitored using the oxidation-sensitive fluorescent probe dihydrorhodamine-123 and a fluorescence microscope. Upon gravistimulation of the primary maize roots, ROS were observed in the lower cortex of the root within 30 min (Fig. 1B). Prolonged gravistimulation of the root also led to the generation of ROS in the lower and upper cortex. This indicates that asymmetric production of ROS in the early stages following gravistimulation could play a role in the gravitropic growth response. To test this possibility further, we investigated the effect of exogenous H_2O_2 on the gravitropic response. Placing H_2O_2-containing agar on the upper side of the root tips inhibited gravitropic curvature, whereas placing the agar on the lower side of the root stimulated gravitropic curvature (Rashotte et al., 2000; Fig. 2A). Furthermore, the asymmetric application of H_2O_2 to the tips of vertical roots induced curvature toward the H_2O_2 source (Fig. 2B).

Scavenging of ROS Inhibits Maize Root Gravitropism

We next assessed the effect of *N*-acetyl-Cys (NAC), an antioxidant, on the gravitropic response. Scavenging of ROS by treatment with 1 mM NAC inhibited root gravitropism (Fig. 3, A and B) without affecting root growth (data not shown). To further strengthen the evidence that asymmetric generation of ROS may play a role in root gravitropism, the effects of asymmetric treatment of the root with NAC were investigated. Placing NAC-containing agar on the upper side of the root enhanced gravitropic curvature rela-

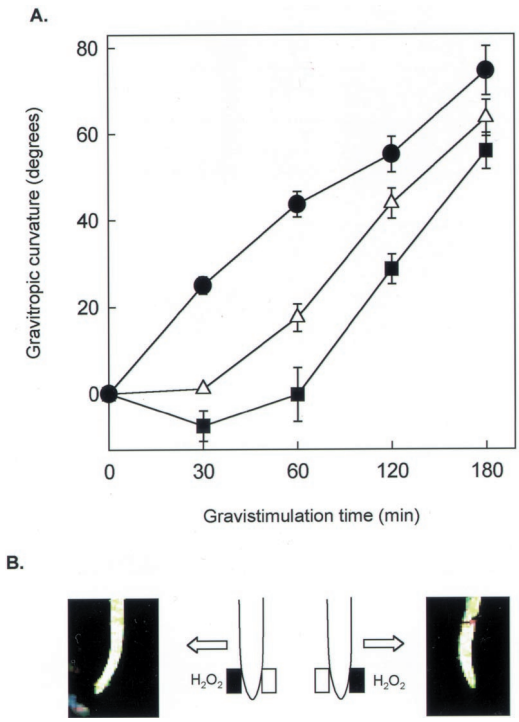

Figure 2. Effect of asymmetric application of H_2O_2 on root curvature. A, Induction of curvature in horizontal roots by asymmetric application of H_2O_2. Agar (1.5%, w/v) blocks were immersed into 1 mM H_2O_2 in 5 mM MES buffer [2-(*N*-morpholino)ethanesulfonic acid, pH 6.8] and then put on the lower side (●) or the upper side (■) of roots held horizontally. △, Control root showing normal gravitropism. Values are the means ± SE for five independent experiments. B, Induction of curvature in vertical roots by asymmetric application of H_2O_2.

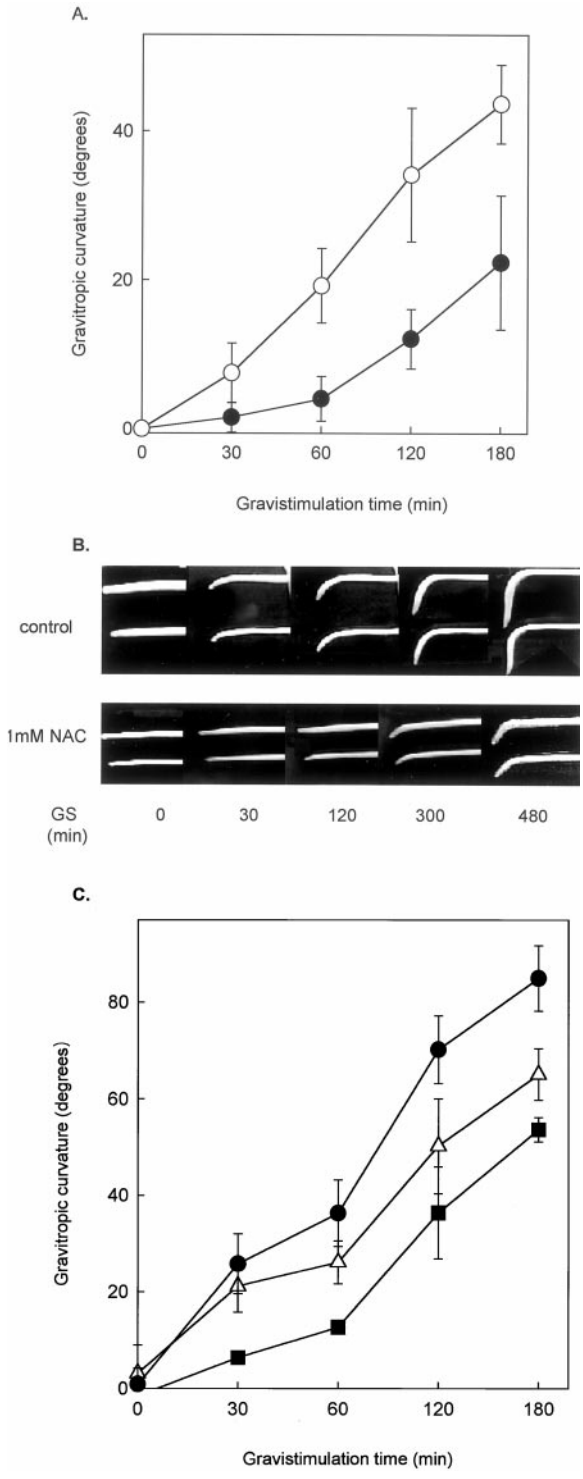

Figure 3. Effect of NAC on gravitropic root curvature. A and B, Suppression of gravitropic curvature by pretreatment with NAC. Roots were immersed in 1 mM NAC for 2 h and then oriented horizontally to induce gravitropism. ○, Control maize roots; ●, NAC-treated roots. C, Induction of curvature by asymmetric application of NAC. Agar (1.5%, w/v) block was soaking into 10 mM NAC solution in 5 mM MES buffer (pH 6.8) and then put on the lower side (■) or the upper side (●) of roots held horizontally. △, Control roots, which were subjected to gravistimulation. Values are the means ± SE for five independent experiments.

tive to controls, whereas placing NAC on the lower side of the root impaired gravitropism (Fig. 3C). We investigated the effect of other antioxidants on gravitropism. Membrane-permeable antioxidants such as 3 mM ascorbic acid and 100 μM trolox, pyrrolidinedithiocarbamate have an inhibitory effect on gravitropism similar to that of NAC (data not shown). However, treatments of the root with catalase, which cannot permeate the epidermal layer of roots, had no effect on the inhibition of gravitropism (data not shown). These results suggest that the ROS are generated inside the cells.

Auxin-Mediated ROS Generation

It is well established that redistribution of auxin plays an important role in plant gravitropism (Young et al., 1990; Rashotte et al., 2000). To test for a possible link between auxin redistribution and generation of ROS in gravistimulated roots, we investigated the effect of applied auxin on ROS generation in the root tip using fluorescence microscopy. Placing indole-3-acetic acid (IAA)-containing agar on one side of the root tip stimulated ROS generation in the treated region (Fig. 4A). We next asked whether auxin can induce ROS generation in root protoplasts directly (Sheen, 1990). The generation of ROS by auxin was measured with DCF-DA and flow cytometry (Bae et al., 1997). Stimulation of the ROS generation in the protoplasts with auxin resulted in a time-dependent increase in the intensity of DCF fluorescence, with the maximal 2.5-fold increase within 10 min after stimulation; fluorescence had returned to the baseline value after 20 min (Fig. 4B). These results suggest that the redistribution of auxin by gravity induces an increase in the gravitropic curvature in maize root through the generation of ROS. NPA is an inhibitor of auxin transport inhibitor and is known to inhibit gravitropism (Rashotte et al., 2000). Therefore, we examined the effect of NPA on the ability of asymmetric application of H_2O_2 to enhance root gravitropism. NPA-treated roots did not show gravitropism. However, placing H_2O_2-contaning agar on the lower side of NPA-pretreated roots induced curvature toward the site of application (Fig. 4C). These results indicate that the action of asymmetrically applied H_2O_2 in causing root curvature does not depend upon auxin redistribution, suggesting that ROS play a role as a downstream component in the auxin-mediated signaling pathway.

It is well established that ROS enhance the phosphorylation and activation of a number of proteins, including mitogen-activated protein kinase (MAPK), in mammalian and plant cells (Sundaresan et al., 1995; Bae et al., 1997; Kotvun et al., 2000). Our current results indicate that gravistimulation elicits a transient increase in intracellular ROS and a rapid phosphorylation of ZmMAPK5 in maize root (data not shown). ZmMAPK5 has sequence homology with

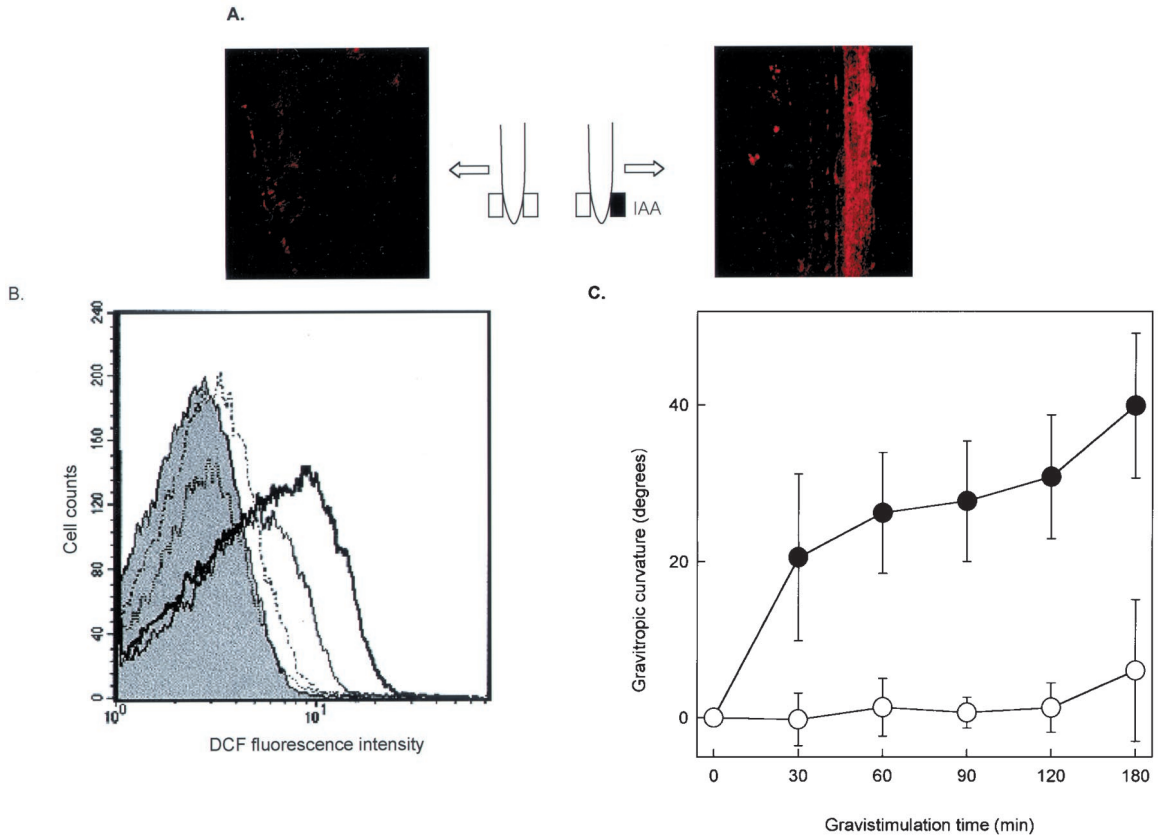

Figure 4. Auxin-induced ROS generation. A, Agar blocks (1.5%, w/v) were incubated in 5 μM IAA in 5 mM MES (pH 6.8) and then placed on the indicated region. ROS generation was detected by fluorescence microscopy. B, Transient generation of ROS by auxin. Intracellular ROS generation in protoplast was measured by 5 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Eugene, OR) and flow cytometry (FACScan, Becton-Dickinson, Bedford, NJ). Shaded area means control fluorescence intensity. Protoplasts were incubated with 5 μM IAA for 5 min (thin line), 10 min (thick line), 20 min (dashed line), or 30 min (dot line). C, Effect of *N*-(1-naphthyl)phthalamic acid (NPA) on ROS-induced gravitropism. Roots were immersed in 5 μM NPA in 5 mM MES buffer (pH 6.8) and then gravistimulated. H_2O_2-containing (●) or control agar (○) was then placed on the lower side of roots held horizontally. Values are the means ± SE for five independent experiments.

AtMAPK6 in Arabidopsis, which is activated by oxidative stress (Kotvun et al., 2000). Furthermore, NAC pretreatmment of roots abolishes the activation of ZmMAPK5 by gravistimulation (data not shown).

Based on the work reported here, we propose a novel role of ROS in plant gravitropism. Gravity induces asymmetric movement of auxin within 60 min, and then the auxin stimulates ROS generation to mediate gravitropism. Several lines of evidence indicate that calcium (Lee at al., 1983) and IP_3 (Perera et al., 1999) as second messengers are also involved in the gravitropic response of roots. Gravitropism stimulated the transient generation of IP_3, which then promoted the opening of an IP_3-induced Ca^{2+} channel to increase intracellular Ca^{2+} (Perera et al., 1999). Furthermore, asymmetric application of exogenous Ca^{2+} caused gravitropic-like curvature of maize roots. How molecular mechanisms involving ROS and Ca^{2+} are integrated into a physiological signal that leads to the gravitropic curvature remains to be elucidated.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Caryopses of maize (*Zea mays* L. cv Golden Cross Bantam) were soaked in distilled water for 12 h before planting. The grains were placed between wet paper towels held between opaque trays mounted in a vertical position and germinated at 28°C ± 1°C in the dark. The primary roots of maize were used 2 d after planting (about 2 cm long).

Assay of ROS

The portion of the root we refer to as zone 1 is the apical 4 mm of the root and includes the root cap, meristem, and the apical half of the root elongation zone. The portion we refer to as zone 2 extends from 4 to 8 mm from the root tip and includes the basal half of the elongation zone. Zone 1 (0–4 mm from root tip) or zone 2 (4–8 mm from root tip) of the gravistimulated primary roots was homogenized in distilled water with a pellet disrupter. Supernatants were

collected by microcentrifugation of the extract. Total ROS concentration was determined by a Bioxytech H_2O_2-560 assay kit. This assay is based on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) by ROS under acidic conditions; the ferric ion binds with indicator dye xylenol orange to form a stable colored complex, which can be measured at 560 nm.

Fluorescence Microscope

For the microscopic measurements, gravistimulated or control roots were bisected longitudinally and stained with 0.003% (w/v) dihydrorhodamine-123 solution for 10 min. Fluorescence intensity of oxidized rhodamine was observed with a fluorescence microscope (Zeiss; excitation = 485 nm, emission = 535 nm; Potikha et al., 1999). Experiments were repeated at least five times with similar results. The selected pictures are parts of the microscopic field, which is representative of the entire field. Photographs were taken with PIXERA visual communication suite (version 1.1.0 for Macintosh operating systems, Pixera, Los Gatos, CA).

Determination of Curvature and Elongation of Maize Root

Curvature and elongation were measured using the digital color camera system (HFG, version 1.1, Yongmacom, Seoul, South Korea) that connected to the computer for the gravitropism measurement. Several pretreated or untreated maize seedlings were mounted in vertical or horizontal position in a clear plastic petri dish under near saturating humidity. Curvature and elongation were recorded and displayed automatically by a custom software program (picture measurement system, version 1.1, Yongmacom).

Preparation of Maize Root Protoplast

Etiolated maize root protoplasts were a modified method as described (Sheen, 1990). Protoplasts were isolated from 2-d-old etiolated maize seedlings. Zone1 (0–4 mm from root tip) of the roots was digested in an enzyme solution containing 2% (w/v) cellulase Onozuka RS (Yakult Pharmaceutical Company, Tokyo), 2% (w/v) Cellulysin (Calbiochem/Behring Diagnostic, La Jolla, CA), 0.026% (w/v) pectolyase Y23 (Sigma, St. Louis), 0.6 M mannitol, 10 mM MES (pH 5.7), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.1% (w/v) bovine albumin (Sigma) for 5 h at 22°C. Protoplasts were separated from the partially digested tissues by passage through a mesh. The protoplast was washed three times with solution of 0.45 M mannitol and 1 mM $CaCl_2$ and stored in the dark.

Flow Cytometry

Protoplasts were incubated with 5 μM auxin. After various incubation times, cells were loaded with 5 μM DCF-DA (Molecular Probes). This compound is converted by intracellular esterases to 2′,7′-dichlorofluorescin, which is then oxidized by H_2O_2 to the highly fluorescent DCF. The fluorescence intensity was measured by FACScan (Becton-Dickinson) with excitation and emission settings of 488 and 530 nm, respectively. Counting of cells stopped at 30,000. Gating was performed prior to the collection of data to remove apoptotic cells and cellular debris. All experiments were repeated three times.

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