1509 Genetic Diversity in Pollen Abiotic Stress Tolerance

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ABSTRACT

Genetic diversity in reproductive abiotic stress tolerance has been investigated by cotton breeders throughout the public and private sectors. The primary focus of these studies has been the evaluation of abiotic stress responses during the development of the flower prior to anthesis. Sterility indices based upon the percentage of anther dehiscence are commonly used as a measure of mature pollen availability in adverse environments. An underlying assumption in these studies has been that the mature pollen released from the anthers is essentially equal among varieties, and that reproductive success is primarily associated with mature pollen availability. We have investigated mature pollen characteristics and have identified distinct genetic variability in the abiotic stress tolerance of the mature pollen. We have developed laboratory-based tests permitting rapid evaluation of the overall abiotic stress tolerance of the pollen. Our findings provide breeders with a previously unexplored reservoir of genetic diversity associated with reproductive abiotic stress tolerance.

Keywords: Cotton, Pollen, Genetic Diversity, Stress Tolerance

Plants experience high air and soil temperatures during periods of drought and when fields receive limited irrigation. These stresses frequently coincide in areas with hot arid climates where one stress can serve to compound the effects of the other. This problem is exemplified in a study comparing dryland and irrigated cotton (*Gossypium hirsutum* L.) that demonstrated that plants grown under water-stress conditions exhibited up to 85% reduction in leaf area index, plant size and dry matter accumulation compared to irrigated controls (Burke et al., 1985a). Dryland cotton leaves endured higher daytime canopy temperatures (40°C) than leaves from irrigated plants (30°C). This effect was due to reduced water availability that resulted in stomatal closure that in turn resulted in leaf temperatures rising above the surrounding air temperature.

Some or all of the negative impact of damaging high temperatures can be ameliorated by prior exposure to elevated, but non-lethal temperatures, in a process called acquired thermotolerance. Plants, like most organisms, respond to an elevation in temperature by synthesizing heat shock proteins (Boston et al., 1996; Miernyk, 1999; Schoffl et al., 1998; Vierling, 1991). Leaves of dryland cotton have been shown to accumulate significant levels of heat shock proteins in response to heat stress (Burke et al., 1985b). During high temperature stress low molecular weight heat shock proteins (15-30 kD in size) can constitute up to 1% to 1.5% of total leaf cellular protein content (Hsieh et al., 1992; Mansfield and Key, 1987).

Pollen has been reported to be sensitive to elevated temperatures (Abdul-Baki and Stommel, 1995; Bajaj et al., 1992; Dane et al., 1991; Halterlein

et al., 1980; Herrero and Johnson, 1980; Iapichino and Loy, 1987; Kakani et al., ; Kuo et al., 1981; Ledesma and Sugiyama, 2005; Matlob and Kelly, 1973; Prasad et al., 2006; Rao et al., 1992; Sakata et al., 2000; Weaver and Timm, 1989), and is the only plant organ that does not synthesize heat shock proteins in response to heat stress (Gagliardi et al., 1995; Hopf et al., 1992; Volkov et al., 2005). Exposure to elevated temperatures during flower development has also been shown to inhibit pollen formation and induce flower sterility (Fisher, 1975). Because of this, reproductive stress responses reported in the literature focus primarily on flower abortion and flower sterility (Percy et al., 2006). Limited information on the genetic diversity in mature pollen heat tolerance is available (Burke et al., 2004; Kakani et al., 2005). These studies evaluated heat stress responses during pollen germination. The present study investigated genetic variability in the abiotic stress tolerance of the mature pollen. Heat stress was imposed on pollen in situ and evaluated in vitro for germination and pollen tube development responses. The importance of humidity levels on pollen viability and germination was also investigated. We have developed laboratory-based tests permitting rapid evaluation of the overall abiotic stress tolerance of the pollen. Our findings provide breeders with a previously unexplored reservoir of genetic diversity associated with reproductive abiotic stress tolerance.

MATERIALS AND METHODS

Greenhouse and field studies were conducted in 2005, 2006 and 2007 at the Plant Stress and Water Conservation Laboratory in Lubbock, TX. The conventionally tilled field plots were treated with Prowl (BASF Corporation, Florham Park, NJ) according to manufacturers instructions for weed control. Individual 15 to 30 meter rows of NM67 and Phytogen 72 were planted in a East-West orientation using a John Deere 7300 MaxEmerge 2 VacuMeter Planter. Replicate plots were planted for irrigated and dryland comparisons. The irrigated plants received 5 mm of water per day per acre from underground drip lines located on 1 m centers. Greenhouse studies evaluated plants whose seeds were planted in Sunshine® 3-Mix soil (Sun Gro Horticulture Canada Ltd, Bellevue, WA) in 5 gallon pots. The pots were well watered using an automated drip irrigation system. Nutrients were maintained by daily application with Peters Excel fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH) through the automated watering system.

Flowers were harvested between 0930 h and 1030 h from the greenhouse plants and placed in ziplock plastic bags for transport into the laboratory. Upon returning to the lab, the flowers were placed on moistened Model 583 Gel Dryer Filter Paper (Bio-Rad Laboratories, Hercules, CA) in a Pyrex baking dish. The flowers and filter paper were covered with Glad® ClingWrap {CO_2 permeable} (The Glad Products Company, Oakland, CA). Replicate samples were placed in the dark in VWR Model 2005 incubators (Sheldon Manufacturing, Inc., Cornelius, OR) set to 39°C or 28°C. The trays containing the flowers were incubated for 5

h, the flowers were then removed from the trays and the pollen collected by gently tapping the inverted flower. The pollen was germinated in vitro at 28°C according to the procedure of Burke (Burke et al., 2004). The pollen was incubated on the media for 1 h prior to analysis. Pollen germination was determined microscopically using a Leica MZ6 modular stereomicroscope (Leica Microsystems Inc, Bannockburn, IL). The percent germination was determined for 16 replicate experiments.

Pollen drying was evaluated in a 25% RH environment. Flowers were harvested between 0930 h and 1030 h from the greenhouse plants and placed in ziplock plastic bags for transport into the laboratory. Upon returning to the lab, the petals of the flowers were removed and the flowers with exposed pollen were placed on a bench top for 6.5 h. Following the treatment, the pollen was germinated in vitro at 28°C according to the procedure of Burke (Burke et al., 2004). The pollen was incubated on the media for 1 h prior to analysis. Pollen germination was determined microscopically using a Leica MZ6 modular stereomicroscope (Leica Microsystems Inc, Bannockburn, IL). The percent germination was determined for 16 replicate experiments.

Pollen germination was evaluated in 25% and 80% RH environments. Flowers were harvested between 0930 h and 1030 h from the greenhouse plants and placed in ziplock plastic bags for transport into the laboratory. Upon returning to the lab, the pollen was collected by gently tapping the inverted flower. The pollen was germinated in vitro at 28°C according to the procedure of Burke (Burke et al., 2004) with half of the pollen placed in a 25% RH environment and

the other half placed in an 80% RH environment. The pollen was incubated on the media for 1 h prior to analysis. Pollen germination was determined microscopically using a Leica MZ6 modular stereomicroscope (Leica Microsystems Inc, Bannockburn, IL). The percent germination was determined for 3 replicate experiments.

The water content of the pollen grains was evaluated according to the procedure of Nepi et al. (Nepi et al., 2001). Fresh pollen was weighed, dried in a 104°C oven, and reweighed to determine the amount of water loss. The percent pollen water content was determined for 5 replicate samples per line.

The rate of water movement into pollen was evaluated by monitoring the time required for the pollen to rupture in an aqueous medium. Flowers were harvested between 0930 h and 1030 h from the greenhouse plants and placed in ziplock plastic bags for transport into the laboratory. Upon returning to the lab, the flower petals were folded back and the anthers dipped into 3-4 drops of a 0.8 M sucrose solution on a glass microscope slide. A cover slip was immediately placed on the slide and the time required for the pollen grains (a minimum of 100 grains per field of view) to rupture was determined microscopically using a Leica MZ6 modular stereomicroscope (Leica Microsystems Inc, Bannockburn, IL). The time to the first pollen grain rupture was determined for 41 replicate flowers. The pollen from field-grown cotton was evaluated using flowers harvest at 0930 h and 1330 h to determine if the time to first rupture changed over time.

RESULTS AND DISCUSSION

Heat sensitivity. Temperature incubations were performed in the presence of high humidity in an attempt to separate temperature stress from humidity responses. The flowers were incubated in a Glad Wrap covered chamber with moistened filter paper on the bottom of the pyrex dish to ensure an elevated humidity level. The results of the 39°C 5 h incubation showed similar reductions of approximately 60 % in pollen germination compared to the 28°C samples in both the NM67 and Phy72 cotton lines (Fig. 1).

In situ pollen dehydration. The drying of the pollen on the anthers was evaluated in the laboratory setting. Flowers with excised petals were placed on the laboratory bench for 6.5 h at 23°C and a 25% relative humidity. Following the drying treatment, pollen was isolated from the flowers and the percent germination evaluated. The germination was expressed as a percent of the germination observed for freshly harvested pollen at the beginning of the experiment. The Phy72 pollen showed a 46% reduction in pollen germination and the NM67 pollen showed a 30% increase in pollen germination following the drying experiment (Fig. 2). It is interesting to note that the initial germination percentages of the two lines following 1 h on the incubation medium were 33% for the Phy72 and only 13% for the NM67 (Fig. 3). The samples following the drying were both at 17% germination. These results suggested the possibility of genetic differences in the pollen's ability to retain internal moisture or in their ability to take up moisture from the in vitro pollen germination medium. Before testing this hypothesis further it was necessary to determine if the pollen from the Phy72 and NM67 had similar moisture contents at the beginning of the study.

Pollen Water Content. Pollen moisture contents were determined by weighing fresh pollen, drying the pollen at an elevated temperature, and weighing the pollen after the moisture had been removed during the drying treatment. Replicate experiments showed equal water contents in the pollen from the Phy72 and NM67 immediately following dehiscence. Both lines exhibited water contents of 51% (Fig. 4). Having shown that the pollen started with equal internal water contents, experiments were performed to determine if pollen tube length development was impacted by the humidity surrounding the pollen during germination.

Humidity Effects on Pollen Tube Development. To evaluate humidity effects, freshly harvested pollen was placed on the in vitro germination medium described by Burke (Burke et al., 2004). The Petri dishes were placed either into an 80% relative humidity chamber or into the incubator having a relative humidity of 25%. The pollen was allowed to germinate and pollen tubes were measured following 1 h of treatment. The tube lengths of the pollen from the 25% RH treatment were compared with the lengths of the tubes from the 80% treatment. The NM67 showed a 38% reduction in pollen tube length when germinated in a 25% RH environment compared with the 80% RH environment (Fig. 5). The PHY72, however, showed a 63% reduction in pollen tube lengths in the 25% RH environment. These results support the hypothesis that the PHY72 may lose internal water more rapidly than the NM67. This water loss affects germination and pollen tube development in vitro.

Pollen Water Uptake. If the assumption that water is lost more readily from the PHY72 pollen than the NM67 pollen is correct, then it is reasonable to hypothesize that water movement into the PHY72 pollen may occur more rapidly that the NM67. This might explain the greater germination percentage of the PHY72 pollen in our controls for the dehydration experiment described earlier. Another way that we chose to test this hypothesis was to evaluate the rate of water uptake into the pollen. This was accomplished by monitoring the swelling and rupturing of the pollen grains in aqueous media. Burke (Burke, 2002) reported the hypersensitivity of cotton pollen to water and that pollen grains placed in water would swell and rupture in seconds to minutes. In order to optimize the detection of genetic differences in pollen water uptake between cotton lines, we evaluated a range of osmotic media to slow the rate of pollen popping and maximize the difference should they exist. We observed optimum differences in the rate of pollen popping using a 0.8 M sucrose solution. A 2-fold difference in the time required to rupture the NM67 pollen was observed when compared to the PHY72 (Fig. 6). A four-minute incubation was required for the first pollen grain to rupture in the NM67 and only 2 min were required for the PHY72. These findings further support the hypothesis that there exists genetic difference in the ability of pollen to retain internal water levels and maintain pollen viability.

Pollen from field-grown plants was evaluated at 0930 h (dehiscence) and at 1330 h to determine if the time to pollen popping changed throughout the day. Genetic differences in the time to pollen popping were observed at both 0930

and 1330 h (Fig. 7). The time to first pollen grain popping was shorter in the afternoon than in the morning. The differences between the lines was greater in the afternoon sample, possibly because of greater water loss in the Phy72 pollen grains during the 4 h exposure to the hot, dry environment in the field.

The results of the present study show genetic differences in the abiotic stress tolerance of cotton pollen. No genetic differences in pollen heat sensitivity were observed. Genetic differences in sensitivity to humidity were observed impacting pollen survival in dry environments. These findings have implications on gene flow from plant to plant that require further investigation.

Figure 1. High temperature sensitivity of cotton pollen from NM67 and Phy72 cotton. Flowers were incubated at 28C or 39C in high humidity chambers for 5 h, the pollen removed, and in vitro germination evaluated at 28C and 80% relative humidity.

Figure 2. In situ pollen dehydration of cotton pollen from NM67 and Phy72 cotton. Flower petals were removed and the exposed anthers and pollen were incubated for 6.5 h in a 25% relative humidity. In vitro pollen germination evaluated at 28C and 80% relative humidity. The percent reduction in pollen germination of the low humidity treated pollen was compared with the germination of freshly dehisced pollen (control).

Figure 3. In situ pollen dehydration of cotton pollen from NM67 and Phy72 cotton. Flower petals were removed and the exposed anthers and pollen were incubated for 6.5 h in a 25% relative humidity. In vitro pollen germination evaluated at 28C and 80% relative humidity. The percent pollen germination of the low humidity treated pollen was compared with the germination of freshly dehisced pollen.

Figure 4. The water content of cotton pollen at dehiscence from NM67 and Phy72 cotton.

Figure 5. Graph of the effect of humidity on in vitro pollen tube length development of cotton pollen from NM67 and Phy72 cotton. Germination at 25% relative humidity was compared with germination at 80% relative humidity.

Figure 6. Graph of the time to first pollen grain rupture when cotton pollen from greenhouse-grown NM67 and Phy72 cotton were placed in 0.8 M sucrose.

Figure 7. Graph of the time to first pollen grain rupture when cotton pollen from field-grown NM67 and Phy72 cotton were placed in 0.8 M sucrose. Pollen samples were evaluated at 0930 and 1330 h.

Figure 1

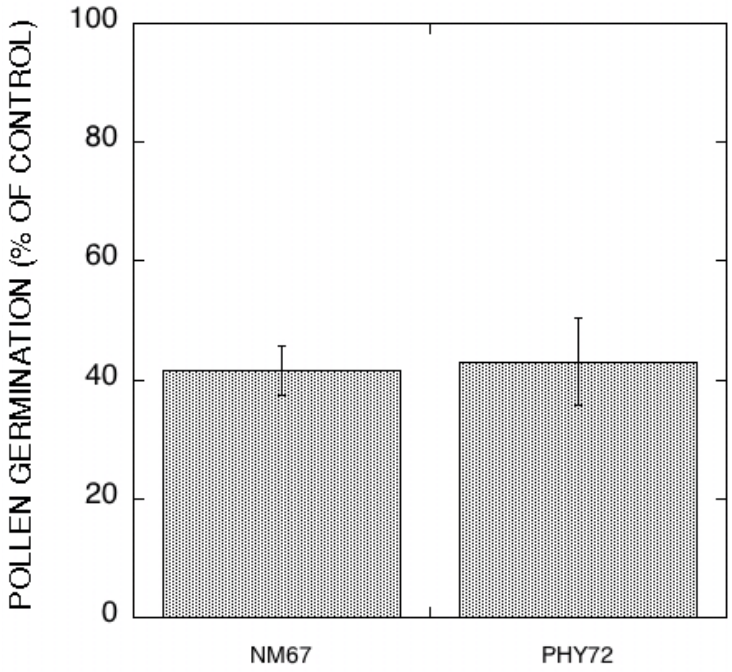


Figure 2.

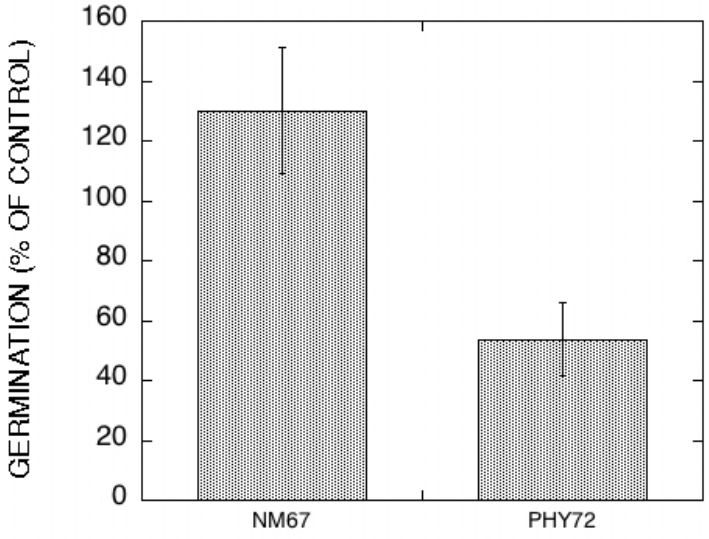

GERMINATION (% OF CONTROL)
160
140
120
100
80
60
40
20
0
NM67
PHY72

Figure 3.

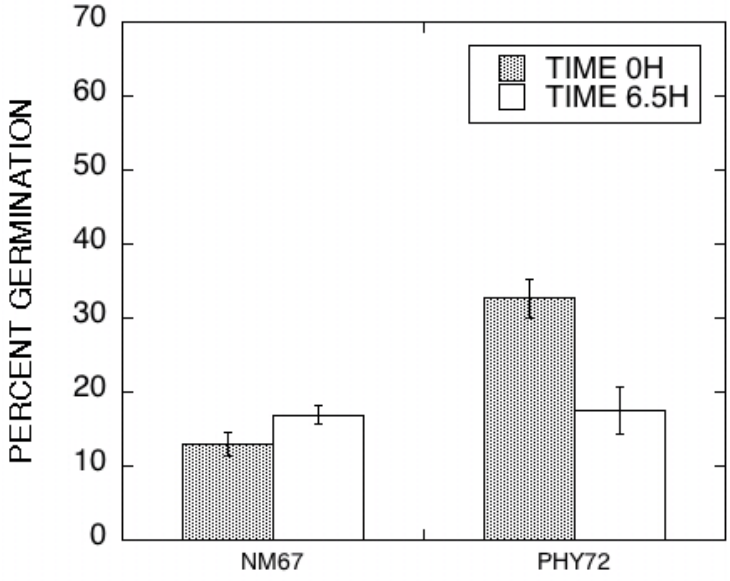

70
60
50
40
30
20
10
0
PERCENT GERMINATION
TIME 0H
TIME 6.5H
NM67
PHY72

Figure 4.

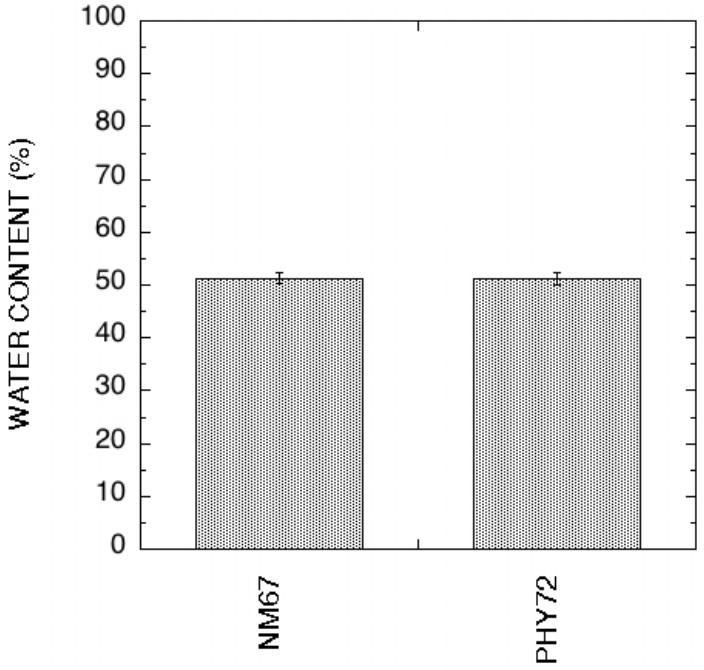


Figure 5.

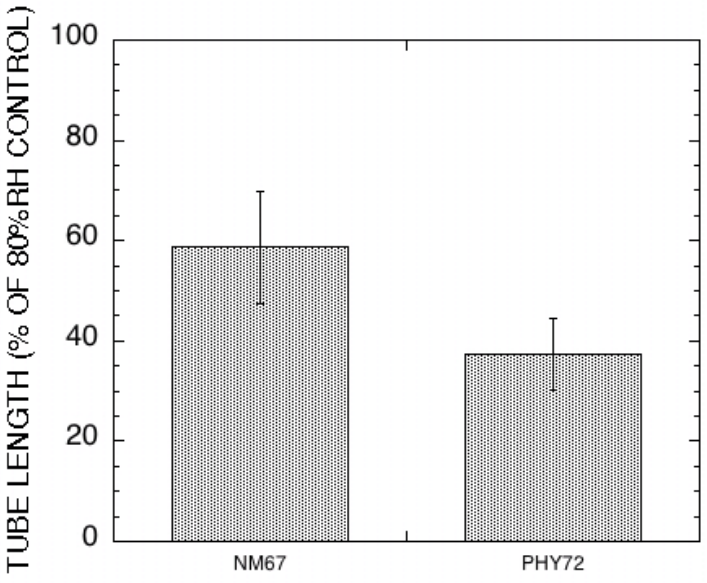


Figure 6.

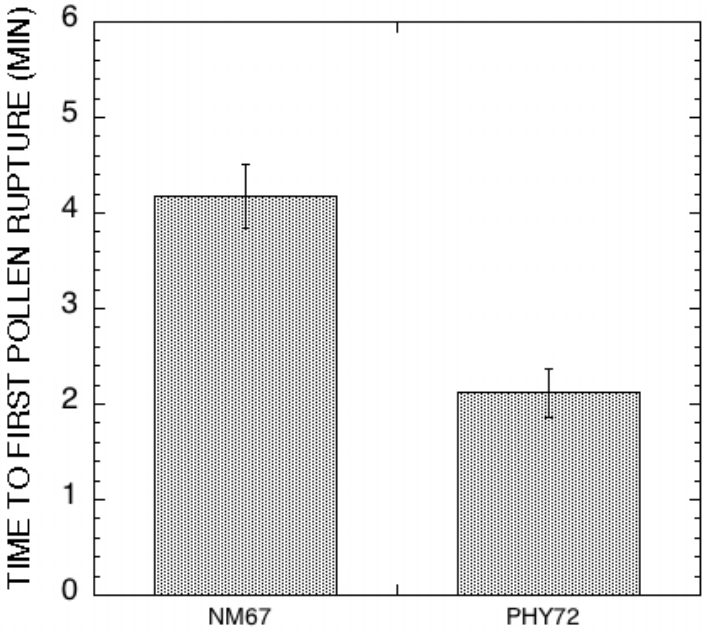
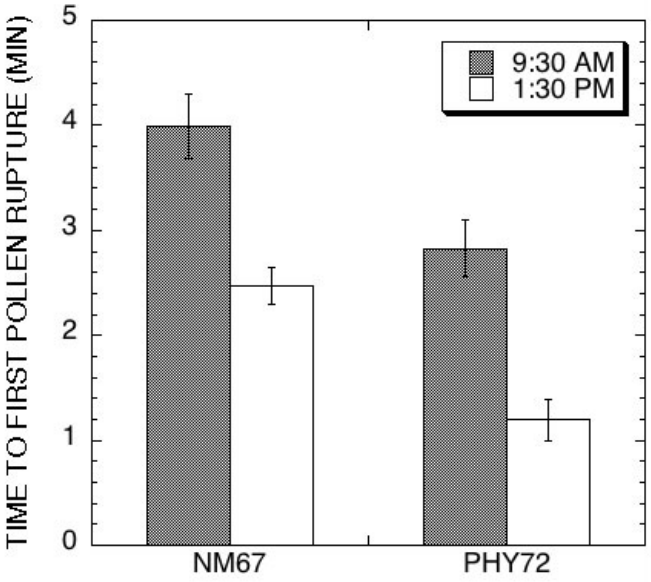


Figure 7.


TIME TO FIRST POLLEN RUPTURE (MIN)
5
4
3
2
1
0
9:30 AM
1:30 PM
NM67
PHY72

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