Plant Maturity and Vernalization Affect Flowering in *Dianthus japonicus* Thunb.

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*Dianthus japonicus* Thunb. (*D. japonicus*) is a biennial with promising floricultural traits, but its commercial appeal is limited by the long time between propagation and flowering. We assessed the effect of juvenile phase, vernalization, and photoperiod on flowering of *D. japonicus*. Plants were grown in a plug until they had acquired nine, 14, or 16 leaf pairs, and then exposed to a vernalization period of 0, 3, 6, or 12 weeks at 5°C. At the end of the vernalization period, plants were transferred to either long-day treatment or short-day treatment for 10 weeks. In *D. japonicus*, the numbers of new nodes and leaves were correlated with the vernalization period. Plant height was correlated with the number of leaf pairs. As the vernalization period lengthened, the plants produced more nodes and leaves regardless of their growth stage. The maximum plant height increase was over 24.7 cm in the plants that had 16 leaf pairs at 10 weeks after the start of the photoperiod treatment, regardless of the photoperiod. Plants with 14 or 16 leaf pairs and a vernalization period of 12 weeks flowered regardless of photoperiod treatment. None of the plants that had been vernalized for less than 12 weeks flowered or produced flower buds. We noted a significant difference in the flowering response among plants based on the number of leaf pairs and vernalization period. We conclude that *D. japonicus* plants must form 14 to 16 leaf pairs before they can respond to vernalization and require at least 12 weeks of vernalization before flowering. This species has a qualitative response to vernalization and is day-neutral.

**Key Words:** biennials, photoperiodism, qualitative vernalization, shorten life cycle.

**Introduction**

A total of 179 and 4,179 species of native plants are distributed in Korea according to the Korea National Arboretum of the Korea Forest Service (2017). This is significantly higher than the United Kingdom (2,000 species) and Denmark (1,500 species) in terms of the number of species per unit area of the country (Kim et al., 2012). However, due to a lack of systematic research on the physiology of native ornamental plants, native wild flowers in Korea have not been developed as commercial varieties.

*Dianthus japonicus* Thunb. (*D. japonicus*) is native to Japan and Korea (Korea National Arboretum, 2015, 2017; United States National Plant Germplasm System, 2019), and the Korean name is ‘Gaet-pae-raeng-i-kkot’ which means seashore pink flower. This plant is an evergreen biennial (Heo et al., 2007; Jung et al., 2008) in the Caryophyllaceae family. The genus *Dianthus* contains horticulturally and commercially important species such as *D. caryophyllus* (carnation), *D. chinensis* (Chinese pink), *D. barbatus* (sweet william), and *D. plumarius* (cottage pink; Nakano and Mii, 1995). The pink, showy flowers produced by *D. japonicus* from July to September make it a potentially valuable horticultural commodity, but its biennial growth habit, requiring two years for cultivation from seedling to flowering, has limited its value to commercial producers (Holley, 2016). Little is known about the regulation of its flower development, as there appears to have been little research on the flowering of *D. japonicus*.

The change from the vegetative to reproductive phase marks a major transition in plant development (Michaels and Amasino, 2000). Meristems that have
been producing vegetative structures switch to producing flowers. In many plant species, the meristem transition from vegetative to reproductive development is irreversible; thus, the proper timing of this transition is crucial for successful reproduction. Consequently, plants have evolved mechanisms to regulate flowering times in response to environmental cues, enabling them to coordinate flowering with the seasons (Michaels and Amasino, 2000). The juvenile stage, during which a plant is insensitive to conditions that promote floral initiation (Bernier et al., 1981) varies among different plant species. For *Brassica campestris*, the juvenile stage is only a few days (Frield, 1968). In contrast, the average juvenile stage in orchids is two to three years (Kim et al., 2011) and in some woody plants is 30 to 40 years (Hackett, 1985). If a plant is prematurely exposed to reproductive conditions before the end of the juvenile stage, it may not be able to support quality flowers and thereby reduce the uniformity of flowering (Cameron et al., 1996). Some biennial plants pass through a juvenile phase before they enter a vegetative adult phase, in which they readily respond to vernalization. *Oenothera erythrosepala* Borbis, in the biennial evening primrose family, must reach a critical size with a 10 cm rosette diameter and 900 mg shoot weight after which it is capable of bolting (rapidly transitioning to flowering). *Verbascum thapsus*, commonly called mulleins and in the biennial plant genus, does not flower until its rosettes are greater than 9 cm in diameter (Gross, 1981). However, it is uncertain if *D. japonicus* has a juvenile phase, although Takeda (1996) observed that *Dianthus* spp. that require vernalization have a juvenile phase that lasts for nine weeks from seeding, at which time 9–10 leaf pairs had been produced. *D. caryophyllus*, carnation, must from 9–11 leaf pairs before it is capable of flowering (Blake, 1955). Flowering of *D. caryophyllus* was promoted when the plant had 16 leaf pairs (Dahab, 1967).

Flowering of many winter annuals, biennials, and perennials is promoted after exposure to low temperatures (Padhye and Cameron, 2008). This phenomenon, known as vernalization, has been defined as a chilling treatment that confers the ability to flower or accelerates flowering. Vernalization may play one of three roles in flowering: plants may have no vernalization requirement, they may respond in a facultative and quantitative manner, or they may require vernalization to flower (qualitative; Padhye et al., 2006). Winter annuals, for example, respond in a facultative manner: flowering does not require cold exposure, but occurs more rapidly after cold treatment (Ha, 2014). Biennials have an obligate requirement, and cannot flower until cold exposure, and insufficient vernalization can result in incomplete or delayed flowering (Padhye and Cameron, 2008). Vernalization requirements within the genus *Dianthus* vary with the species (Chouard, 1960). For example, *D. barbatus* ‘Kyakko’ requires no vernalization to flower, while *D. Mikadonadeshiko* ‘Miss Biwako’ achieved 13%, 73%, 87%, and 93% flowering in vernalization periods of 0, 3, 6, and 9 weeks, respectively (Takeda, 1996). *D. barbatus* ‘Arima’ required nine or more weeks of vernalization.

In many plant species, floral transition is precisely controlled by photoperiod and temperature (Li et al., 2016). Recent advances in molecular genetics in model plants revealed that flowering signals from the photoperiod and vernalization pathway are integrated into the *FLOWERING LOCUS T* (*FT*) gene, a major component of florigen and flowering time QTL associated with *FLC paralogues* (Imaizumi and Kay, 2006; Xiao et al., 2019). Photoperiod and developmental stages converge to regulate the expression of *FT*, a major gene in leaves. Lower temperature-dependent flowering regulation has been characterized and temperature also regulates *FT* in leaves (Song et al., 2013).

Here, we studied the flowering response to developmental stages and vernalization in *D. japonicus*. Specifically, we investigated the effect of plant age at the time of transfer to inductive conditions on flowering, and identified the optimal inductive conditions, focusing on vernalization (using chilling treatments) and photoperiod. We aim to determine an accurate flowering time for *D. japonicus*, and to suggest a protocol that will shorten its flowering time to less than two years, permitting successful commercial production.

**Materials and Methods**

*D. japonicus* seeds were grown in a greenhouse in a plug tray (5.7 cm × 5.7 cm × 6.5 cm/cell; Bummong Tray, Junguep, Korea) initially until they reached different vegetative growth stages of nine, 14 and 16 leaf pairs. Plants with nine leaf pairs had 7.5 nodes and were 6.1 cm tall, those with 14 leaf pairs had 7.9 nodes and were 7.2 cm tall, and those with 16 leaf pairs had 9.1 nodes and were 7.3 cm tall. To confirm specific maturity for plants with nine, 14, and 16 leaf pairs, they were grown in a greenhouse for 12, 16, 20 weeks after sowing on the 13th May, 7th April, and 17th March, 2015, respectively. The plants were grown in the greenhouse until 20th August. The average greenhouse temperatures were 9.8°C, 13.3°C, 18.9°C in March, April, and May, respectively. The relative humidity was 50 ± 5% in this period. From June to August, the average greenhouse temperature was 25.3°C (maximum temperature: 30.2°C and minimum temperature: 21.6°C), and the relative humidity was 65 ± 5%. When plants in the greenhouse reached the indicated stages, they were transplanted into 10 cm diameter plastic pots filled with commercial potting medium (Sunshine Mix #1; Sun–Gro Horticulture, Bellevue, WA, USA). After transplanting from plug trays to plastic pots, the plants were moved from the greenhouse to a growth chamber (HB–303DHC; Hanbaek Scientific Co., Suwon, Korea) for vernalization treatment. The plants were supplied with
tap water until they were transplanted. Nutrients were supplied for plants when they were exposed to vernalization treatment. The plants were fertilized once a month with a nutrient solution (15N–15P–18K, electrical conductivity of 1.0 dS·m$^{-1}$; Eco special series, HYPONeX Japan Co., Osaka, Japan). Vernalization was performed at 5°C for 0, 3, 6, or 12 weeks, respectively. Light in the growth chamber was supplied by white fluorescent lamps (AL–2220D; A–Lim Industrial Co., Ltd., Incheon, Korea) for 12 h (05:00–17:00) during the vernalization period. Plants grown for 0, 3, and 6-week vernalization periods were grown with 12 h (05:00–17:00) light provided by white fluorescent lamps (AL–2220D; A–Lim Industrial Co., Ltd., Incheon, Korea) and a constant temperature of 25°C and for non-vernalization after the vernalization treatment for 12, 9, and 6 weeks, respectively. The purpose of this treatment was to make a total of 12 weeks with vernalization and non-vernalization periods for all plants.

After a 12-week vernalization (or non-vernalization) period, plants were randomly assigned to either short-day (SD) or long-day (LD) conditions for 10 weeks in a growth room with a constant temperature of 25°C. Light was supplied by white fluorescent lamps (FLR40EX–W/A; Osram Korea, Ansan, South Korea) for an average light intensity of 56–60 μmol·m$^{-2}$·s$^{-1}$, assessed by line quantum sensors (Apologee Instruments, Inc., Logan, UT, USA). The photoperiod of SD treatment was 10 h (07:00–17:00). The LD condition was created by night interruption (NI) lighting at 22:00 to 02:00 in addition to a photoperiod of SD treatment with light from white fluorescent lamps (AL–2220D; A–lim Industrial Co., Ltd., Incheon, Korea) at an intensity of 4–7 μmol·m$^{-2}$·s$^{-1}$. NI can be an effective strategy with low light intensity to apply LD for as long as day-extension, for example in *Dianthus chinensis* (Kim et al., 2011; Park et al., 2013).

Plant vegetative growth characteristics were assessed weekly during the experimental period. New leaves were counted as such if they were 0.5 cm or longer. New nodes were also counted. Plant height was measured from the soil to the uppermost shoot, then height increases were calculated. Bolting was considered to have occurred when the flower stalk was 10 cm or longer, assessed as the percentage of plants that had bolted in each treatment group. Flowering was defined as full expansion of the first flower. At flowering, the number of floral buds that were 0.5 cm long or longer was counted for each plant.

The experimental design was a randomized complete block with three replications with four plants in four pots. Three-way analysis of variance (ANOVA) was performed to test the effects of pairs of leaves, vernalization period, and photoperiod and means were compared by the Tukey–Kramer honest significant difference (HSD) test, at $P < 0.05$, using the software package SAS system for Windows version 9.4 (SAS Inst. Inc., Cary, NC, USA). Regression and graph module analyses were performed using Sigma Plot software (version 10.0; Systat Software, Inc., Chicago, IL, USA).

**Results**

The plants with 14 or 16 leaf pairs and a vernalization period of 12 weeks flowered by the end of the 10 weeks after vernalization (Fig. 1). None of the plants that had been vernalized for less than 12 weeks bolted or flowered (Figs. 1 and 2). Plants with 14 leaf pairs at the start of the 12 weeks of vernalization bolted before the end of the experiment, and those grown in LD...
bolted sooner than those in SD. Plants with 75% of 14 leaf pair groups that were grown in SD bolted. In the group of plants with 16 leaf pairs at the start of the 12 weeks of vernalization, however, the plants in SDs bolted before the plants in LDs (Fig. 2).

As the vernalization period increased, the plants produced more nodes and leaves during the subsequent 10-week growth period, regardless of their vegetative growth stage when the vernalization treatment was started (Table 1). More new nodes and leaves were produced in the plants vernalized for 12 weeks compared with the shorter vernalization periods in both photo-periods. The plants with nine leaf pairs produced at most 8.3 new nodes during the 10-week growth period, regardless of photoperiod (Table 1), while during this time period, plants that were at the 14 and 16 leaf pairs stage produced an average of 12.7 and 15.5 nodes after 12 weeks of vernalization, respectively. In all plants with 14 and 16 leaf pairs, the numbers of new nodes and new leaves were significantly higher ($P < 0.05$) in the 12-week vernalization period than at 0, 3, and 6 weeks. There were no significant differences ($P < 0.05$) in the number of new nodes or new leaves according to the photoperiod.

In general, leaf and node production in the two most mature groups of plants that were exposed to 12 weeks of vernalization showed a large height increase compared to the plants exposed to shorter vernalization periods (Fig. 1; Table 1). In contrast, the height during the 10-week growth period did not increase in the plants with nine leaf pairs after vernalization for 12 weeks. The maximum height increase was 27.4 cm in the plants that had 16 leaf pairs at the start of the 12-week vernalization period and LD treatment, and the height increase was approximately 24.0 cm greater than the plants with nine leaf pairs and 12 weeks of vernalization, regardless of photoperiod. Numbers of new nodes, leaves, and height increase were not significantly different in the plants exposed to the same vernalization period but grown under different photoperiod conditions.

The plants that had bolted or flowered had more leaf pairs than plants that had not bolted (Fig. 2; Table 2). Only the plants with 14 or 16 leaf pairs and a vernalization period of 12 weeks flowered by the end of the 10 weeks after vernalization (Fig. 1; Table 2). The plants with nine leaf pairs that received 12 weeks of vernalization did not flower during the time of the experiment. There was no significant difference in the number of floral buds or flowers produced by the plants with respect to photoperiod ($P < 0.05$). More of the plants with 16 leaf pairs flowered than the plants with 14 leaf pairs (Table 2).

A three-way ANOVA showed that the main factors and their interactions had significant effects on the total

![Fig. 2](image)
number of floral buds and flowers (Table 2). The total numbers of floral buds and flowers of *D. japonicus* were significantly affected by the vernalization period (*P* < 0.001), but not by the photoperiod. Leaf pairs of *D. japonicus* significantly affected the total number of floral buds and flowers between the number of leaf pairs and vernalization period (*P* < 0.001). There were no significant interactions between plant maturity and photoperiod, or between vernalization period and photoperiod. Significant interactions were observed in the total number of floral buds and flowers between plant maturity, vernalization period, and photoperiod (*P* < 0.01).

### Discussion

Plants with a qualitative cold response generally require a period of vegetative growth before they can respond to the cold temperature stimulus (Cave and Johnston, 2010). Exposing plants to reproductive conditions before the end of the juvenile stage can result in negative effects on flowering (Cave et al., 2011). A common method for determining the length of the juvenile phase is to transfer plants from growth conditions to stimulating conditions at regular intervals during

### Table 1. Vegetative growth parameters of *Dianthus japonicus* Thunb. that were transferred to different vernalization periods and photoperiods when they were at different plant maturity stages (as measured by number of leaf pairs). Measurements were made 10 weeks after the start of the photoperiod treatment.

| Plant maturity^z^ | Vernalization period (weeks) | Photoperiod | Number of new nodes | Number of new leaves | Height increase (cm) |
|-------------------|-----------------------------|-------------|---------------------|---------------------|---------------------|
| 9                 | 0                           | LD^y^       | 1.5 e               | 3.8 f               | 0.1 c               |
|                   |                              | SD          | 2.8 de              | 4.8 ef              | 0.4 c               |
|                   | 3                           | LD          | 5.3 c-e             | 5.5 d-f             | 0.1 c               |
|                   |                              | SD          | 1.8 e               | 5.8 d-f             | 0.1 c               |
|                   | 6                           | LD          | 5.5 c-e             | 6.8 d-f             | 0.1 c               |
|                   |                              | SD          | 3.3 c-e             | 7.0 b-f             | 0.1 c               |
|                   | 12                          | LD          | 7.3 b-d             | 10.0 b-d            | 2.1 c               |
|                   |                              | SD          | 8.3 bc              | 10.0 b-d            | 2.1 c               |
| 14                | 0                           | LD          | 4.8 c-e             | 8.5 d-f             | 0.1 c               |
|                   |                              | SD          | 3.0 c-e             | 9.0 c-e             | 0.1 c               |
|                   | 3                           | LD          | 3.3 c-e             | 7.8 d-f             | 0.2 c               |
|                   |                              | SD          | 3.3 c-e             | 6.5 d-f             | 0.1 c               |
|                   | 6                           | LD          | 4.5 c-e             | 9.0 c-e             | 1.1 c               |
|                   |                              | SD          | 2.0 de              | 8.3 d-f             | 0.4 c               |
|                   | 12                          | LD          | 13.8 a              | 13.5 ab             | 18.8 ab             |
|                   |                              | SD          | 11.5 ab             | 15.8 a              | 13.2 b              |
| 16                | 0                           | LD          | 4.3 c-e             | 8.5 d-f             | 0.1 c               |
|                   |                              | SD          | 3.3 c-e             | 8.3 d-f             | 0.1 c               |
|                   | 3                           | LD          | 4.0 c-e             | 8.3 d-f             | 0.3 c               |
|                   |                              | SD          | 3.3 c-e             | 6.8 d-f             | 0.1 c               |
|                   | 6                           | LD          | 6.8 b-e             | 9.5 c-e             | 0.9 c               |
|                   |                              | SD          | 4.8 c-e             | 9.8 cd              | 0.7 c               |
|                   | 12                          | LD          | 15.5 a              | 14.0 ab             | 27.4 a              |
|                   |                              | SD          | 15.5 a              | 18.3 a              | 24.7 a              |

^z^ Number of leaf pairs at start of vernalization.

^y^ Photoperiod treatments (LD, long-day; SD, short-day).

^x^ Means with the letters in a column are significantly different according to the Tukey–Kramer HSD test (*P* < 0.05). NS, *, and *** indicate not significant or significant at *P* < 0.05 and 0.001, respectively, by three-way ANOVA (n = 4).
plant development and assess subsequent leaf production and flowering. We performed this type of study here. Plants of *D. japonicus* with 9, 14, or 16 leaf pairs were vernalized for 0, 3, 6, or 12 weeks. We conclude that this species was in its “adult” stage when the plant had 14–16 leaf pairs, based on our finding that vernalization of 12 weeks promoted flowering in plants at this developmental stage, but not when the plants had nine leaf pairs. Furthermore, the plants that were older at the time of vernalization produced more floral buds and had flowers than younger plants.

Many biennial plants have a critical size for flowering (Kachi and Hirose, 1983). A relationship between plant age and response to vernalization that is similar to our findings with *D. japonicus* has been noted in *Coreopsis grandiflora*, *Gaillardia × grandiflora*, and *Rudbeckia fulgida*: the juvenile phase of these plants ends when they have about 8, 16, or 10 nodes, respectively (Yuan et al., 1998). Baskin and Basin (1979) concurred that most biennials have a critical size for flowering and suggest that this requirement allows vegetative growth to proceed while conditions are favorable. Older plants may be more floriferous than younger plants as older plants will likely have greater vegetative mass at the start of vernalization, with more photosynthetic capacity to support flowers (Cave et al., 2011).

### Table 2.

Flowering parameters of *Dianthus japonicus* Thunb. that were transferred to different vernalization periods and photoperiods when they were at different plant maturity stages (as measured by number of leaf pairs). Measurements were made 10 weeks after the start of the photoperiod treatment.

| Plant maturitya | Vernalization period (weeks) | Photoperiod | Total number of floral buds | Total number of flowers | Flowering percentage (%) | Days to flowering |
|----------------|-------------------------------|-------------|-----------------------------|-------------------------|--------------------------|------------------|
|                |                               |             | 0.0 d                       | 0.0 c                   | —                        | —                |
| 9              | 0 LD                          | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
|                | 3 LD                          | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
|                | 6 LD                          | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
|                | 12 LD                         | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
| 14             | 0 LD                          | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
|                | 3 LD                          | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
|                | 6 LD                          | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
|                | 12 LD                         | SD          | 14.8 bc                     | 6.3 b                   | 50.0 62.0                | 62.0             |
| 16             | 0 LD                          | SD          | 8.3 cd                      | 3.8 bc                  | 50.0 61.5                | 61.5             |
|                | 3 LD                          | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
|                | 6 LD                          | SD          | 0.0 d                       | 0.0 c                   | —                        | —                |
|                | 12 LD                         | SD          | 22.8 a                      | 14.8 a                  | 100.0 61.0               | 61.0             |

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* Number of leaf pairs at start of vernalization.

* Days to flowering was investigated in plants with over 50% flowering.

* Photoperiod treatments (LD, long-day; SD, short-day).

* Means with the letters in a column are significantly different according to the Tukey–Kramer HSD test (*P* < 0.05). NS, **, and *** indicate not significant or significant at *P* < 0.01 and 0.001, respectively, by three-way ANOVA (n = 4).

* The hyphen symbol (—) indicates that the plants did not flower at the end of the experiment.

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Vernalization has been defined as the acquisition or acceleration of the ability to flower by a chilling treatment. The period of vernalization has a quantitative effect on the flowering response until it is maximal, and therefore, increasing the period of vernalization progressively increases flowering up to a point (Lang, 1965; Padhye and Cameron, 2008). Insufficient vernalization can result in incomplete or delayed flowering (Padhye and Cameron, 2008). The effective vernalization period for promotion of flowering is species-specific. D. gratianopolitanus, ‘Bath’s Pink’, flowered after 15 weeks of treatment at 5°C (Padhye and Cameron, 2008). In this study, D. japonicus plants that had been vernalized at 5°C for 0, 3, or 6 weeks did not flower, while plants vernalized for 12 weeks at 5°C did flower. Also, height increased as the vernalization period was extended in all plants, regardless of their maturity (number of leaf pairs) at the start of vernalization. Increasing plant height is the first visible sign of the switch from vegetative to generative development. Therefore, we concluded that at least 12 weeks of vernalization are necessary for induction of bolting in D. japonicus.

Vernalization responses of plants can be qualitative or quantitative (Padhye and Cameron, 2008). Plants with a qualitative vernalization response require cold treatment to flower. In plants with a quantitative vernalization response, cold treatment accelerates flowering or improves flowering characteristics. The response to vernalization is specific to species of Dianthus: D. granicicus and D. barbatus have a quantitative response, while D. arenarius, D. caryophyllus, and D. gallicus have a qualitative response (Chouard, 1960). D. japonicus has been previously reported to have no cold or daylength requirement for flowering (Takeda, 1996). In this study, however, we observed that the biennial D. japonicus failed to flower unless vernalized for 12 weeks. Thus, we conclude that vernalization is a requirement of this species, and furthermore, it has a qualitative response to vernalization.

Plants can be divided into several groups regarding photoperiod response: SD, LD, and day-neutral (DN) plants (Ha, 2014). DN plants flower regardless of the daily light period. Based on our results, we suggest that D. japonicus is day neutral. Plants with 14 or 16 leaf pairs that had been vernalized for 12 weeks flowered in both LDs and SDs, and the number of flower buds and flowers produced by the plants was not significantly different in the two photoperiods, and the photoperiod did not affect the time it took for the plants to bolt or the percentage of plants that flowered. All of these results confirm our conclusion that D. japonicus is day neutral.

The flowering characteristics of D. japonicus can be summarized as follows. Plants must have formed 14 to 16 pairs of leaves before they can respond to vernalization and require at least 12 weeks of vernalization. D. japonicus has a qualitative response to vernalization and is a DN plant. It appears that Dianthus japonicus Thunb., with proper management, can flower in as little as 46 weeks after sowing. We hope the information in this study will assist in optimizing flowering in D. japonicus and contribute to the introduction of this new Dianthus cultivar to the floriculture market.

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