Infection of *Tomato spotted wilt virus* (TSWV) shortens the life span of thelytokous *Thrips tabaci* (Thysanoptera: Thripidae)

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Abstract

The effect of tomato spotted wilt virus (TSWV) infection on the life span of thelytokous *Thrips tabaci*, which is known as a TSWV vector with a low transmission rate, was studied in two populations, Shimane (SM) and Iwate (IW). No effects of virus infection were found in the developmental period and mortality of the thrips before adult emergence, but a significant increase was observed in age-specific mortality during the adult lifespan of the TSWV-exposed thrips group when compared with the non-exposed group (mean total longevity of thrips exposed and not exposed to virus was 18.1 and 20.1 d in the SM population and 19.9 and 21.5 d in the IW population). The latent period (LP) was 14.2 d in the SM population and 17.2 d in the IW population, indicating a relatively longer LP than the LPs reported for *Frankliniella occidentalis* and *F. fusca*. The potential transmission period (PTP) from the end of the LP to vector death was only 3.3 d in both of the populations. The higher the level of virus infection, the greater the reduction in adult thrips survival. These results suggest that a long LP and TSWV-induced reduction of thrips survival shorten the PTP. This may be responsible for the low transmissibility of TSWV as well as the low transmission rate in thelytokous *T. tabaci* populations.

Key words: *Thrips tabaci*; *Tomato spotted wilt virus*; parthenogenesis; mortality; virulence

INTRODUCTION

Tomato spotted wilt virus (TSWV), the type species of the genus *Tospovirus*, family *Bunyaviridae* (Murphy et al., 1995), is one of the most alarming viruses infecting many agricultural and horticultural crops and is the cause of severe epidemics worldwide (OEPP/EPPO, 1999, 2004; Sherwood et al., 2001; Ullman et al., 2002). The virus is exclusively transmitted by seven species of thrips (Thysanoptera: Thripidae) in a persistent manner (Sherwood et al., 2001; Ullman et al., 2002). Only thrips that acquire the virus in the young larval stage become transmitters, either as old larvae or as adults (Smith, 1931; Linford, 1932; Sakimura, 1963; Ullman et al., 1992; Wijkamp and Peters 1993; van de Wetering et al., 1996), after a latent period of several days, wherein the virus replicates and circulates in the vector host (Ullman

et al., 1993; Wijkamp et al., 1993). The viruliferous thrips appear to be able to retain the transmission ability of the virus for life (Sakimura, 1962; Wijkamp et al., 1996).

The onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), is not only a ubiquitous species prevalent worldwide as an agricultural pest causing serious economic losses, but also a vector of TSWV (Smith, 1931; Linford, 1932; Sakimura, 1962; Anon., 1969; Lewis et al., 1997). Although *T. tabaci* has been recognized as a primary vector of TSWV for many decades (Smith, 1931; Linford, 1932; Sakimura, 1962, 1963), their inefficiency and failure in transmission of TSWV have been recently reported (Wijkamp et al., 1995; Chatzivassiliou et al., 2002; Inoue et al., 2004a). Currently, the vector status of *T. tabaci* is relatively low when compared with other vector species such as *Frankliniella occidentalis*, *Frankliniella schultzei*

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(Wijkamp et al., 1995; Inoue et al., 2004a; Sakurai, 2004). In addition, distinct transmission rates were reported in their reproductive mode, host preference and among geographic populations (Chatzivassiliou et al., 2002). Thelytokous populations (a virgin female produces only female offspring) have no or low vector competence, whereas arrhenotokous populations (an unmated female produces only male offspring) possess a certain level of competence (Wijkamp et al., 1995; Tedeschi et al., 2001; Chatzivassiliou et al., 2002; Inoue et al., 2004a).

The vector competence may be strongly affected by the longevity as reported in the mosquito-*Plasmodium* relationship (Dye, 1992; Carey, 2001): a longer longevity might lead to greater disease transmission during the adult stage, while a shorter longevity might lead to less disease transmission. The negative effects of TSWV on the life history traits of thrips vector might also lead to a low vector competence for thelytokous *T. tabaci*.

The vector competence for TSWV has been mostly regarded as the transmission rate for a certain period, for example, for 6 d after adult emergence using the petunia leaf disc assay (Wijkamp et al., 1995; Chatzivassiliou et al., 2002). However, it is likely that thrips maintain the ability for their entire life (Sakimura, 1963; Wijkamp et al., 1996). Therefore, vector competence should also be considered as the lifetime transmission frequency during the potential transmission period (PTP) from the terminal of the latent period (LP) (i.e., the onset of transmission) to the vector death.

To test whether TSWV infection affects the longevity of thelytokous *T. tabaci*, we compared the development time, pre-adult mortality and adult age-specific mortality between virus-exposed and unexposed cohorts. We also calculated the length of the PTP from longevity data and the LP, and then analysed the effects of the intensity of TSWV infection on the PTP and the lifetime transmission frequency.

MATERIALS AND METHODS

Thrips. Two thelytokous populations of *T. tabaci* were used in the present study. The populations were located on the Japanese main island; one population was collected from onions, *Allium cepa* L. (Liliaceae), in Shimane Prefecture (35°22'N, 132°45'E) (hereafter referred to as SM)

in 1996, and the other population was collected from leeks, *Allium ampeloprasum* L. (Liliaceae), in Iwate Prefecture (39°44'N, 141°08'E) (hereafter referred to as IW) in 2001. These populations have been maintained using germinated broad beans, *Vicia faba* L. (Fabaceae), and tea pollen, *Camellia sinensis* (L.) Kuntze (Theaceae), at $23 \pm 0.5^\circ\text{C}$ and a L16:D8 photoperiod (for details see Murai and Loomans, 2001). Voucher specimens of both populations were deposited in the Laboratory of Insect Resources, Faculty of Agriculture, Tokyo University of Agriculture.

Virus isolate. A TSWV isolate (TSWV-Iw02) obtained in 2002 from infectious tomato, *Lycopersicon esculentum* Mill. (Solanaceae), in Iwate Prefecture, Japan, originated from a single local lesion of TSWV after three mechanical inoculations and was maintained on *Datura stramonium* L. (Solanaceae) by inoculation with viruliferous thrips or mechanical inoculation. The amino acid and the nucleotide sequences of the nucleocapsid (N) protein of this isolate showed 98.4% and 97.2% identity, respectively, to those of the Brazilian isolate BR-01 (de Haan et al., 1990; Inoue et al., 2004b).

Virus acquisition by thrips. Approximately half of the newly hatched larval cohorts, 0–8 h old, was allocated to a detached *D. stramonium* leaf that was systemically infected with TSWV, while the other half was allocated to a healthy leaf. Each procedure was performed in a 66 mm-diameter Petri dish, and the top of the Petri dish was sealed with a stretched laboratory film for an acquisition access period (AAP) of 16 h at $23 \pm 0.5^\circ\text{C}$ and L16:D8. TSWV uptake and infection in thrips were confirmed 0 h and 72 h after AAP by a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The larvae were reared on healthy germinated broad beans at $23 \pm 0.5^\circ\text{C}$ and L16:D8 until adult emergence.

Development period and pre-adult mortality. The developmental period and mortality from the post-TSWV acquisition period to adult emergence in virus-exposed and unexposed cohorts were compared using a leaf cage method (for details, see Inoue et al., 2004a). After acquisition as described above, 12 to 18 larvae were placed on a soybean leaf, *Glycine max* (L.) Merr., sandwiched between two acryl plates until adult emergence. The leaves were replaced with fresh ones every 2 d. The devel-

opment of thrips was checked every day and dead thrips were removed. After pupation, each cohort was monitored for 8 h to determine adult emergence. In the distinct number of thrips tested per repetition in this study, no effects were observed in the developmental period and mortality.

Adult mortality. Age-specific mortality was examined using the cohorts whose newly hatched larvae (simultaneously hatched) were exposed and unexposed to TSWV. During the development, the cohorts were maintained using germinated broad beans, *Vicia faba*. After emergence, thrips that were randomly chosen from 15 to 40 adults of every population per trial were transferred individually into a 1.5 ml transparent microtube (Assist, Inc., Tokyo) containing a leaf disc of *Petunia* × *hybrida* cv. Polo Blauw (Wijkamp and Peters, 1993) with a diameter of 6 mm and a piece of 25 mm²-filter paper for the prevention of excess humidity. The survival and mortality of the thrips were scored at 24 h intervals, and the leaf discs were replaced daily with fresh ones. The observation was continued until all of the thrips died. The dead thrips were stored at -80°C until DAS-ELISA. The missing samples were regarded as censored observations, which are terminated before the occurrence of the event (Allison, 2004), on analyses between exposed and unexposed cohorts. All experiments were carried out at 23 ± 0.5°C and L16:D8.

TSWV transmission, LP, and PTP. The petunia leaf discs used in the experiment for measuring the mortality of thrips were floated on distilled water for 72 h in a 96-well ELISA plate to observe symptom development. Infection of leaf discs expressing local lesions was checked by a simplified rapid immunofilter paper assay (RIPA) (Tsuda et al., 1992; Ohki and Kameya-Iwaki, 1996) using commercially available immunostrips (Agdia, Inc., USA). Leaf discs from healthy plants were used as controls. The transmission rate of TSWV in the thrips population was calculated as the percentage of individuals that had developed local lesions to the leaf discs. The latent period (LP) was determined as the time from the end of AAP to the first successful transmission. The vector competence of thrips for TSWV has been regarded as the frequency of thrips transmitted virus in a population for a certain period (e.g. Wijkamp et al., 1995; Chatzivassiliou et al., 2002). However, it is likely

that transmitter thrips retain the ability for life (Sakimura, 1963). Therefore, we should consider vector competence as the lifetime transmission frequency. We counted the lifetime transmission frequency for all of the thrips tested: the thrips were transferred individually into a 1.5 ml microtube containing a petunia leaf disc and the leaf discs were replaced daily with fresh ones. Thus, the lifetime transmission frequency is the total frequency counted by day. Moreover, we determined the period from the onset of transmission to the death of thrips for the virus-transmitted thrips and referred to this period as the potential transmission period (PTP).

Detection of viral accumulation from thrips. To detect internal virus accumulation, thrips were analysed individually using DAS-ELISA with monoclonal antibody (0.25 µg/ml) raised against the N protein of TSWV and an alkaline phosphatase conjugated antibody (0.25 µg/ml) (Agdia, Inc., USA). In the substrate step, the reaction was allowed to proceed for 30 min at room temperature. The absorbance values were measured at 405 nm (A_{405}). Six healthy thrips were added to each plate as negative controls. Blank values were read for wells without thrips in the sample incubation step. The ELISA values were corrected by subtracting the mean of the blank absorbance values from the sample values. The level of virus accumulation was regarded as intensity of virus infection (i.e. infectivity).

Statistics. Statistical analyses were performed using open source software, R, version 2.0.0 (R Development Core Team, 2004; available at <http://www.r-project.org/>). The developmental period and pre-adult mortality of the TSWV-exposed thrips were compared with those of the unexposed thrips using the Mann-Whitney *U*-test and Fisher exact test, respectively (Zar, 1999). The relationship between virus infectivity and death time on TSWV-exposed thrips was analysed using Spearman rank correlation (Zar, 1999).

We estimated the survival curves for exposed and unexposed cohorts with the Kaplan-Meier method, which is appropriate for continuously censored event times (Allison, 2004). The potential differences between the two treatment groups in survival distributions were tested by the log-rank test (Vernables and Ripley, 2002). The effect of virus infection on the mortality of thrips (hazard

ratio, $\exp(\beta)$) was analysed by the Cox proportional hazard model (Cox, 1972; Parmar and Machin, 1995). The hazard ratio for the exposed cohort was estimated as the instantaneous risk of death relative to that of the unexposed control. Handling of the tied data (two or more observations with the same event time) in Cox's model was performed using Efron's approximation (Allison, 2004) because of the large number of ties. The likelihood ratio test was performed to determine whether the regression coefficient is zero (Lee and Wang, 2003); the value of zero would suggest that there is no effect of virus infection on survival.

RESULTS

Virus acquisition and accumulation in larval thrips

Two *T. tabaci* populations showed a high TSWV uptake immediately after an AAP of 16 h with virus-infected *D. stramonium*. The rate of virus acquisition from these populations was 91.3% ($n=46$; A_{405} 0.510 ± 0.034 , mean $\pm$ SE) in SM and 90.2% ($n=41$; A_{405} 0.590 ± 0.035) in IW. Subsequently, there was high TSWV accumulation in most of the second larval individuals grown on virus-free germinated bean pod: the rate of viruliferous thrips was 90.0% ($n=30$; A_{405} 0.866 ± 0.089 , mean $\pm$ SE) in SM and 100% ($n=30$; A_{405} 1.246 ± 0.095) in IW. These observations, therefore, implied successful virus acquisition in almost all thrips.

Developmental period and pre-adult mortality

Development time was not significantly different between the virus-exposed and unexposed cohorts in both SM and IW populations (Table 1). Although the mortality during the development of thrips for the virus-exposed cohort was higher—from 6.4% to 12.4%—than for its unexposed counterpart, no significant differences were observed (Table 1).

Adult longevity and age-specific mortality

The mean adult longevity ($d \pm$ SE) of the virus-exposed cohort was significantly shorter than that of the unexposed cohort. The exposed and unexposed cohorts showed values of 18.1 ± 0.3 and 20.1 ± 0.4 , respectively, in SM and 19.9 ± 0.3 and 21.5 ± 0.4 , respectively, in IW (Log-rank test,

Table 1. Larva-to-adult development time and mortality of thelytokous *Thrips tabaci* exposed and unexposed to Tomato spotted wilt virus (TSWV)^{a,b}

Population Treatments	Development time ^c (days after AAP)	Mortality ^d (%)
SM		
Exposed	9.9 ± 0.1 ($n=54$)	16.9 ($n=65$)] NS
Unexposed	9.6 ± 0.1 ($n=51$)	
IW		
Exposed	10.2 ± 0.1 ($n=52$)	18.8 ($n=64$)] NS
Unexposed	9.9 ± 0.1 ($n=44$)	

^a 0 to 8-h-old larvae were divided into two cohorts. They were given an acquisition access period (AAP) of 16 h to either the TSWV-infected *Datura stramonium* plant or a healthy plant.

^b The total number tested for TSWV-exposed and unexposed thrips was 66 and 60 thrips, respectively, in the SM population and 66 and 48 thrips, respectively, in the IW population. However, 1 out of 66, 3 out of 60, 2 out of 66 and 1 out of 48 thrips, respectively, were missing during the experiments; therefore, these samples were ruled out from the analysis.

^c The values (mean $\pm$ SE) obtained from only survival thrips were analysed using the Mann-Whitney *U*-test. NS designates not significant at $p > 0.05$.

^d The values were calculated from the total number of thrips tested, excluding the thrips missing from the experiment. The differences between treatments in mortality were analysed using the Fisher exact test. NS designates not significant at $p > 0.05$.

$\chi^2_1=17.0$, $p<0.0001$ for SM; $\chi^2_1=9.2$, $p<0.01$ for IW) (Fig. 1). The regression coefficient β of the Cox proportional hazard model was estimated at $0.533 (\pm 0.130 \text{ SE})$ and $0.428 (\pm 0.144 \text{ SE})$ for SM and IW populations, respectively (Likelihood ratio test, $\chi^2_1=17.8$, $p<0.0001$ for SM; $\chi^2_1=8.94$, $p<0.01$ for IW; Efron ties handling) (Fig. 1). The estimated hazard ratio for the virus-exposed thrips was 1.70 ($=\exp(0.533)$) and 1.53 ($=\exp(0.428)$) times higher than that for the unexposed thrips in SM and IW populations, respectively. Adult mortality of the exposed cohort was negatively correlated with virus infectivity (Spearman rank correlation coefficient, $r_s=-0.4123$, $n=206$, $p<0.0001$ for SM; $r_s=-0.3735$, $n=111$, $p<0.0001$ for IW) (Fig. 2).

LP, PTP, and TSWV transmission

The mean LP was 14.2 ± 0.3 ($d \pm$ SE, $n=65$) for SM and 17.2 ± 0.5 ($d \pm$ SE, $n=12$) for IW. The LP was not correlated with virus infectivity (Spearman

Fig. 1. Analysis of survival and mortality curves of thelytokous adult *Thrips tabaci* exposed and unexposed to *Tomato spotted wilt virus* (TSWV) at the young larval stage. The total number of tested exposed thrips and unexposed controls was 213 and 93 thrips, respectively, from the SM population and 114 and 93 thrips, respectively, from the IW population. However, 7 out of 213, 2 out of 93, 3 out of 114 and 1 out of 93 thrips were missing during the experiments. Five to seven repetitions were carried out for each treatment in the experiments.

rank correlation coefficient, $n=65$, $p>0.05$ for SM; $n=12$, $p>0.05$ for IW) (Fig. 3a, b). The estimation of the PTP of transmitter was approximately 3.3 d in both tested populations (Fig. 3c, d). The PTP tended to fall with a rise in virus infectivity in both populations despite of the absence of a statistical decrease in IW (Spearman rank correlation coefficient, $r_s=-0.4648$, $n=65$, $p<0.0001$ for SM; $r_s=-0.4398$, $n=12$, $p>0.05$ for IW) (Fig. 3c, d). The transmitter rate was relatively high when adult thrips were subjected to successive inoculation access periods until death: the SM and IW populations showed values of 31.6% ($n=206$) and 10.8% ($n=111$), respectively (Fig. 2). The minimum ELISA value of the transmitter was 0.302 for SM and 0.393 for IW (Fig. 2). The lifetime transmission frequency of the transmitter thrips was 2.4 ± 0.2 ($\pm$ SE, $n=65$) for SM and 2.5 ± 0.3 ($\pm$ SE, $n=12$) for IW (Fig. 3e, f). The lifetime transmission frequency was liable to be negatively correlated with virus infectivity when it was restricted at

Fig. 2. Relationship between the mortality of thelytokous adult *Thrips tabaci* and infectivity of *Tomato spotted wilt virus* (TSWV) after *T. tabaci* was exposed to the virus at the young larval stage. The total number plotted for TSWV transmitters (+) and nontransmitters (○) was 65 and 141, respectively, from the SM population and 12 and 99, respectively, from the IW population.

least to the transmitter of the SM population (Spearman rank correlation coefficient, $r_s=-0.4049$, $n=65$, $p<0.0001$ for SM; $r_s=-0.3924$, $n=12$, $p>0.05$ for IW) (Fig. 3e, f).

DISCUSSION

A few studies have reported the neutral effects of TSWV on the life-history traits of thrips vectors. No difference was observed in the total longevity between the virus-infected and non-infected *T. tabaci* (Sakimura, 1963), nor in the survival time, developmental time and reproduction between cohorts of virus-infected and uninfected *F. occidentalis* (Wijkamp et al., 1996). These findings suggest that TSWV virulence, i.e. pathogen-induced mortality (Bull, 1994; Read, 1994), is lacking in the thrips vector. In the present study, the development time as well as mortality during the develop-

Fig. 3. Relationships between infectivity of *Tomato spotted wilt virus* (TSWV) and three parameters—latent period (LP) (a, b), potential transmission period (PTP) (c, d), and lifetime transmission frequency (e, f)—in thelytokous adult *Thrips tabaci* after thrips were exposed to the virus at the young larval stage. Only transmitters were plotted for the LP, PTP and lifetime transmission frequency ($n=65$ for SM, $n=12$ for IW).

mental stages of thelytokous *T. tabaci* were likely to be unaffected by virus exposure (Table 1). On the other hand, the longevity was decreased by exposure. TSWV-exposed *T. tabaci* showed a 70% and 53% higher risk of adult mortality than unexposed thrips in the SM and IW populations, respectively (Fig. 1).

The vector competence is strongly affected by the vector longevity (Dye, 1992; Carey, 2001); thus, it should be considered in respect to the PTP in addition to the transmitter rate. The PTP of a species and/or population that is longer than that of others suggests that the chances of virus transmission could increase and vice versa. In the present study, the PTP was estimated at 3.3 d for the SM and IW populations, and the transmitter rates were 31.6% and 10.8%, respectively. Here, if the effi-

ciency score of virus transmission is calculated as transmitter rate $\times$ PTP, the rates for the SM and IW populations are 1.04 ($=0.316 \times 3.3$ d) and 0.36 ($=0.108 \times 3.3$ d), respectively. The score shows that the vector competence of the SM population is 2.9 times higher than that of the IW population. This score is also available for a comparison between vector species. The score of an *F. occidentalis* population is estimated using the data from Wijkamp et al. (1996), and the resultant value is 10.59 ($=0.79 \times 13.4$ d). Thus, the vector competence of *F. occidentalis* would be 10–29 times higher than those of the tested *T. tabaci* populations, although there were differences in the AAP, thermal conditions, etc. This finding appears to be consistent with the present situation that *F. occidentalis* is a primary vector species worldwide in TSWV epidemiology (Ullman et al., 2002). Thus, the long PTP may contribute to the transmission advantage of TSWV.

The relatively long LP may delay the onset of transmission and shorten the total PTP, thereby discounting the lifetime transmission frequency of thrips. The LP of *T. tabaci* is probably longer than that of *Frankliniella fusca* and *F. occidentalis* in TSWV transmission, although the LP is influenced by temperature conditions: 10.7 and 9.3 d, unaccounted for temperature, in American *T. tabaci* and *F. fusca* populations (Sakimura, 1963); 5 d at 24°C in a Dutch *F. occidentalis* population (Wijkamp and Peters, 1993); and 12.9 d at 24°C in a Greek *T. tabaci* population (Chatzivassiliou et al., 2002). Our data showed a mean LP of 14.2 ± 0.3 and 17.2 ± 0.5 (d $\pm$ SE) in SM and IW populations (Fig. 3a, b). The PTP of *T. tabaci* for TSWV transmission is probably shorter than that of *F. fusca* and *F. occidentalis* unless *T. tabaci* is alive for a longer period than *F. fusca* and *F. occidentalis* in particular. Thus, the LP would affect the PTP and vector competence. The distinct length of LP observed in the tested populations may be responsible for the access and multiplication rate of TSWV in the salivary gland because successful transmission requires heavy infection of this organ (Nagata et al., 1999). Our observation showed that the transmission rate of the IW population was lower than that of the SM, implying that TSWV multiplication in the salivary gland is insufficient or is delayed in the IW population as compared to the SM group. Consequently, it may lead to a delay in the LP terminal

(i.e., the onset of virus transmission) in the IW population.

The intensity of TSWV infection is likely to affect successful transmission in thelytokous adult *T. tabaci* in at least two aspects. The previous studies report that the level of infection in thrips transmitted TSWV or *Impatiens necrotic spot virus* is relatively high (e.g., Sakurai et al., 1998, 2004; Inoue et al., 2004a). In the present study, TSWV was also transmitted by the thrips with over a certain level of ELISA value (A_{405}): 0.302 for SM and 0.393 for IW (Fig. 2). On the other hand, the heavy infection of virus led to a reduction in the PTP and the lifetime transmission frequency of virus-transmitted thrips (Fig. 3c–f). This may be due to TSWV-induced mortality from heavy infection (Fig. 2). Therefore, intermediate levels of TSWV infection in thrips might lead to successful transmission in thelytokous *T. tabaci*.

Although it is unclear how the reduced longevity in TSWV-infected thrips will take place, possible mechanisms may be tissue damage and cost of immune response as reported in other insect vector-pathogen interactions (e.g., leafhopper-*Spiroplasma*, mosquito-*Plasmodium*), whereby the pathogens circulate and multiply within the vector (Fletcher et al., 1998; Ferguson and Read, 2002). The pathogen appears to elicit an immune response in the insect vector (Basset et al., 2000). A recent study demonstrated immunities in TSWV-infected *F. occidentalis* (Medeiros et al., 2004). If such immunity exists and trades off survival in thelytokous *T. tabaci*, the heavy TSWV-infection may cause an increase in immune activation, which might lead to a lower survival rate. Further information is required to confirm whether there will be an adverse effect of TSWV infection on other thrips species, populations and arrhenotokous *T. tabaci* and to explain whether such immunity will come at a cost to thelytokous *T. tabaci*.

In the present study, we showed that TSWV infection impaired the longevity of thelytokous adults of *T. tabaci*. The untimely death induced by a high concentration of TSWV and longer LP might limit the PTP, leading to a reduction in lifetime transmission frequency. This may explain the aspect of the low competence of TSWV transmission by thelytokous *T. tabaci* populations.

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