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Review

Testing the genetic autonomy of the plasmon in the *Triticum–Aegilops* complex: The final work of Prof. Koichiro Tsunewaki

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Summary Prof. Tsunewaki dedicated his entire scientific career toward understanding the genetic effects and the diversity of "plasmon", the whole cytoplasmic genetic system, in the common wheat *Triticum* and its relative *Aegilops*. He concluded his life's work with a set of final experiments to test the genetic autonomy of the plasmon in this complex. They were represented by two interconnected studies. First, the examination of the persistence of genetic effects of *Ae. caudata* plasmon on the phenotype of the common wheat, *Triticum aestivum* strain "Tve" (genome: AABBDD) during 63 generations of repeated backcrosses with Tve pollen. The second study involved the reconstruction of an *Ae. caudata* strain by replacing the nuclear genome of the alloplasmic Tve mentioned above, (*caudata*)-Tve SB_{50}, with the genome (CC) of *Ae. caudata*. In this experiment, he tested whether there are any differences between the native plasmon of *Ae. caudata* and the *caudata* plasmon that had remained in common wheat for more than 60 generations. This paper reviews the outline and background of the last work of Prof. Koichiro Tsunewaki.

Keywords Plasmon, Genetic autonomy, *Triticum–Aegilops* complex, Alloplasmic wheat, *Aegilops caudata.*

Prof. Koichiro Tsunewaki had devoted nearly two thirds of his lifetime to wheat genetics, particularly toward the understanding of the genetic diversity and evolution of the "plasmon" in the *Triticum–Aegilops* complex.

Kihara and Fukasawa were the pioneers of the plasmon analysis in the *Triticum–Aegilops* complex in the 1950s. Kihara introduced the plasmon from *Aegilops caudata* (2*n*=14, genome CC) into the common wheat, *T. aestivum* subsp. *aestivum* var. *erythrospermum* (strain "Tve", 2*n*=42, genome AABBDD, hereafter referred to as Tve) (Kihara 1951). Two years later, Fukasawa introduced the plasmon from *Ae. geniculata* (2*n*=28, genome $M^{o}M^{o}UU$, taxonomic synonym: *Ae. ovata*) into a tetraploid wheat, *T. turgidum* subsp. *turgidum* conv. *durum* (2*n*=28, genome AABB, synonym: *T. durum*) (Fukasawa 1953). Both researchers found that the introduced plasmons caused cytoplasmic male sterility in the wheat genotypes, demonstrating the existence of a strong interaction between the genome and the plasmon.

After working as a postdoctoral research fellow in Canada, Prof. Tsunewaki joined Kihara's laboratory at the National Institute of Genetics, Mishima, Japan in 1959. In there, he got an opportunity to work with alloplasmic wheat lines carrying the plasmon of *Ae. caudata*, *Ae. geniculata*, and *T. timopheevi*. As he recalled himself later, this chance encounter spurred interest in him to conduct systematic studies on plasmon diversity and phylogeny in the *Triticum–Aegilops* complex. Prof. Tsunewaki generated a total of 552 alloplasmic lines by combining 12 common wheat genotypes with 47 alien plasmons of *Triticum–Aegilops* (Tsunewaki *et al.* 1996). These studies were conducted in collaboration with Dr. S. S. Maan in USA and Dr. I. Panayotov in Bulgaria. Using these alloplasmic lines, he conducted large-scale systematic studies and deciphered an overall picture of plasmon differentiation and evolution in this complex (for review, see Tsunewaki 2009).

Maternal inheritance of plasmon is well-known in angiosperms (Gillham 1994, Birky 2008). However, the question of whether the plasmon is independent of the coexisting genome (genetic autonomy), had not been investigated in detail until recently. No systematic studies were available, barring an initial study by Michaelis (1965) on a few *Epirobium* alloplasmic lines he generated to study the genetic autonomy of its plasmon.

Prof. Tsunewaki conducted a systematic study to experimentally verify if plasmons are genetically autonomous from the coexisting genome in *Triticum* and

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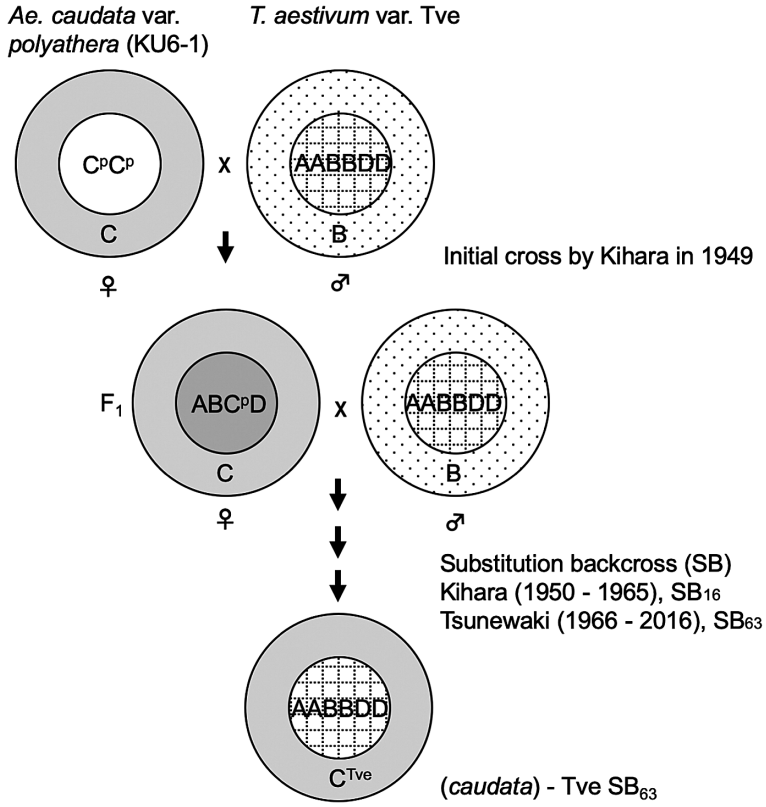

Fig. 1. Production and maintenance of the alloplasmic common wheat, (*caudata*)-Tve SB_{63}, carrying the plasmon of *Ae. caudata* var. *polyathera*. The outer circle and inner circles represent the plasmon and the nuclear genome, respectively. To distinguish the plasmon of native *Ae. caudata* var. *polyathera* from that of (*caudata*)-Tve SB_{63}, the latter is designated as C^{Tve}.

Aegilops, which he decided to be the final experiments in his lifelong work on the plasmon of these plants (Tsunewaki *et al.* 2019).

Both the chloroplast and mitochondrion possess their own genome; however, their functions on the phenotype are often expressed through the interaction between their genes and those of the coexisting nuclear genome. In Prof. Tsunewaki's words: "genetic autonomy of the plasmon does not mean that its function is independent of the genome, but rather that its replication and transmission are independent of control by the nuclear genome".

To test the genetic autonomy of the plasmon, he designed the following two interconnected studies: 1) He selected the alloplasmic common wheat line that carries the plasmon of *Ae. caudata* var. *polyathera* (KU6-1), referred to as (*caudata*) -Tve SB_{63}, which was initially developed by Kihara in 1949 (Kihara 1959) and later continued to be investigated by Tsunewaki for 63 generations through substitution backcross (Fig. 1). Here, SB_{63} indicates the 63rd substitution backcross generation. Using this line, he examined the persistence of the genetic effects on the plasmon of *Ae. caudata* on the phenotype of common wheat strain Tve during 63 generations of repeated backcrosses with Tve pollen. 2) Next, he attempted and eventually succeeded in reconstructing *Ae. caudata* var. *polyathera* by replacing the nuclear genome of the alloplasmic Tve, (*caudata*)-Tve SB_{50}, with the genome of *Ae. caudata* var. *polyathera* (KU6-1). In this experiment, he aimed to reunite the *caudata* plasmon that had resided in common wheat for 50 generations (and in seven other strains for 12 additional generations) with the native genome (CC) of *Ae. caudata* var. *polyathera*. The aim of the study was to test if there are any differences between the native and reconstructed *Ae. caudata* strains. Here I will provide an overview of Prof. Tsunewaki's last study on the genetic autonomy of the plasmon in *Triticum* and *Aegilops*, in which I had the opportunity to work with him.

Testing the persistence of the genetic effects of Ae. caudata *plasmon during 63 generations of repeated backcrosses with the common wheat strain Tve*

A series of systematic investigations was carried out on the performance of all 551 alloplasmic lines with the 12 euplasmic control lines (Tsunewaki *et al.* 2002). These studies examined the phenotypic effects of alien plasmons on 21 traits of wheat to characterize each plasmon. Based on the results, it was shown that the typical effects of the *Ae. caudata* plasmon on the common wheat strain Tve are the induction of male sterility and the production of germless grains (Kihara and Tsunewaki 1964, Tsunewaki *et al.* 2002).

Therefore, to test the genetic autonomy of plasmon, Tsunewaki *et al.* (2019) evaluated the male sterility,

germless grains, and an additional 19 traits of the (*caudata*) -Tve SB_{63} line. The study confirmed that the genetic effects of the *Ae. caudata* plasmon on the phenotype of Tve were limited to the induction of male sterility and the production of germless grains, and these effects did not change during the 63 generations of backcrossing.

Reconstructing the Ae. caudata *var.* polyathera *strain from the genome of the native* Ae. caudata *var.* polyathera *strain and its plasmon resident for 50 generations in common wheat*

The second experiment aimed at reconstructing the *Ae. caudata* var. *polyathera* which carries the *caudata* plasmon that had remained in the AABBDD genome for over half a century. Although it seemed relatively straightforward, this experiment turned out to be the most challenging work and took more than 10 years to complete. (Tsunewaki *et al.* 2019).

Initially, Prof. Tsunewaki crossed the alloplasmic Tve line carrying the *caudata* plasmon, (*caudata*)-Tve SB_{50}, with the pollen of *Ae. caudata* var. *typica* and generated (*caudata*^{Tve})-ABC^{t}D F_1 plants. Here '(*caudata*^{Tve})' refers to the *Ae. caudata* plasmon that resided in the common wheat Tve for 50 generations, and 'C^t' refers to the nuclear genome derived from *Ae. caudata* var. *typica* (KU6-2). Unfortunately, the (*caudata*^{Tve})-ABC^{t}D F_1 plants exhibited complete female sterility, and no SB_1 seeds were obtained from the 1,522 florets backcrossed with *Ae. caudata* pollen. He then generated an alloplasmic octoploid, (*caudata*^{Tve})-AABBC^{t}C^{t}DD, through colchicine treatment of the above F_1 plants. However, even after backcrossing 294 florets with the *Ae. caudata* pollen, no seeds were obtained. This was probably due to an unusual genomic ratio between the nuclei involved in the double fertilization.

Forced to change the strategy, Prof. Tsunewaki decided to use *Ae. cylindrica*, a tetraploid species with C^{c}C^{c}D^{c}D^c genome constitution, for bridging the transfer of the (*caudata*^{Tve}) plasmon from (*caudata*^{Tve})-AABBC^{t}C^{t}DD to *Ae. caudata* (Fig. 2) (Tsunewaki *et al.* 2014). (*caudata*^{Tve})-AABBC^{t}C^{t}DD was crossed with the pollen of *Ae. cylindrica* and (*caudata*^{Tve})-AABBC^{t}C^{c}DD^c F_1 hybrids were obtained. This cross was also difficult and the success rate of the cross [calculated as (seed set rate)×(germination rate)] was 7.2%. The F_1 hybrids were then backcrossed with *Ae. cylindrica* and the SB_1 plants were obtained. This backcross was also very difficult, and the success rate was only 2.3% (only 86 seeds were obtained from 2,656 florets backcrossed, and the germination rate was 71.1%). SB_1 plants with the genome constitution (*caudata*^{Tve})-C^{c}C^{c}D^{c}D^c and accordingly the somatic chromosome number of $2n$=28 or, at worst, $2n$=29 was expected to be obtained. Surprisingly, however, no plants with $2n$=28 or $2n$=29 were obtained

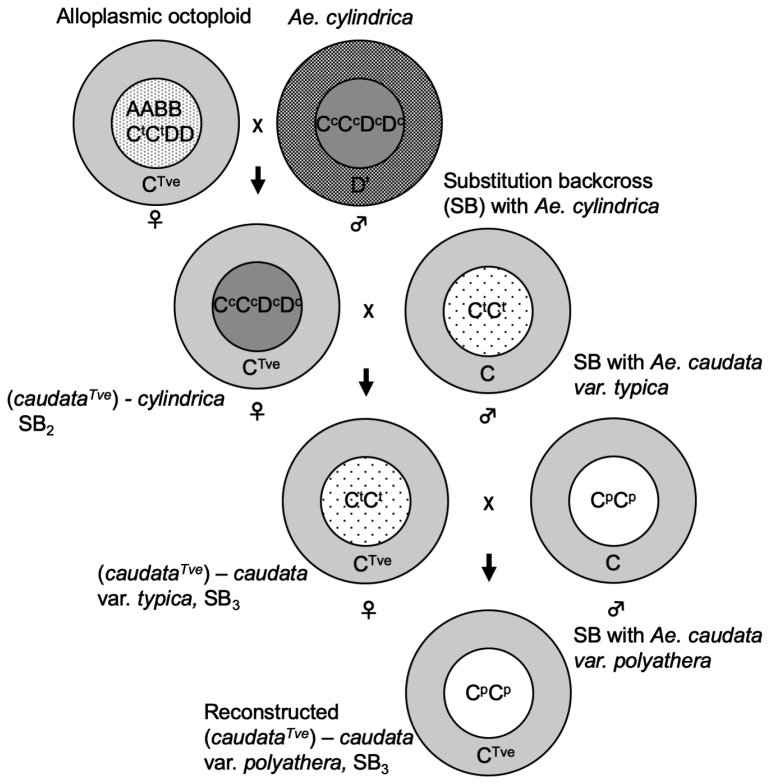

Fig. 2. Reconstruction of the *Ae. caudata* var. *polyathera* strain from the genome of the native *Ae. caudata* var. *polyathera* strain and its plasmon resident for 50 generations in the common wheat variety Tve. To distinguish the CC genome of *Ae. cylindrica*, *Ae. caudata* var. *typica* and *Ae. caudata* var. *polyathera*, their genomes are designated as C^c, C^t and C^p, respectively. Similarly as above, the D genomes of common wheat and that of *Ae. cylindrica* are denoted as D and D^c, respectively.

(chromosome number of SB_1 plants varied from $2n$=32 to $2n$=40). Therefore, it took another year to obtain SB_2 plants that formed 14 bivalents in 46% of their pollen mother cells (PMCs). These SB_2 plants were crossed as the females with *Ae. caudata* var. *typica*, and F_1 plants of (*caudata*^{Tve})-$C^cC^tD^c$ were obtained. This was again another challenging cross with a success rate of only 2.3%. These F_1 hybrids were then backcrossed twice with the pollen from *Ae. caudata* var. *typica* and the SB_2 progeny, (*caudata*^{Tve})-C^tC^t, which showed normal meiosis and full fertility was obtained. The SB_2 plants were backcrossed again and the SB_3 progeny was obtained (Fig. 2).

At this stage, the reconstruction of *Ae. caudata* seemed to have been completed. However, Prof. Tsunewaki found that the original *Ae. caudata* strain used by Kihara (1959) as the plasmon donor to (*caudata*)-Tve was not the var. *typica* (KU6-2), but it was actually *Ae. caudata* var. *polyathera* (KU6-1). Therefore, it became necessary to replace the *Ae. caudata* var. *typica* genome (C^tC^t) with that of the var. *polyathera* (C^pC^p) (Fig. 2), which took another four years. Finally, the genetically stable SB_3 strain of (*caudata*^{Tve})-C^pC^p, *i.e.*, (*caudata*^{Tve})-*caudata* var. *polyathera*, was obtained and the reconstruction was completed. In this (*caudata*^{Tve})-*caudata* var. *polyathera* strain, the genome of *Ae. caudata* var. *polyathera* was successfully reunited with the plasmon (*caudata*^{Tve}), which had been separated from it for more than 60 generations (50 generations in common wheat Tve, and additional 12 generations in seven other strains including *Ae. cylindrica*).

Twenty-two phenotypic traits were compared between the native and reconstructed *Ae. caudata* var. *polyathera* strain, and no significant differences (p<0.05) were detected between the two (Tsunewaki *et al.* 2019). In addition, simple sequence repeat (SSR) loci in both chloroplast and mitochondrial genomes were analyzed using the native and reconstructed *Ae. caudata* var. *polyathera*, as well as the stable strains produced during the course of the reconstruction of the *Ae. caudata* var. *polyathera* strain. The experiment showed that the native *Ae. caudata* var. *polyathera*, (*caudata*)-Tve SB_{60}, (*caudata*^{Tve})-synthetic octoploid, (*caudata*^{Tve})-*Ae. cylindrica* SB_3, (*caudata*^{Tve})-*caudata* var. *typica* SB_2, (*caudata*^{Tve})-*caudata* var. *polyathera* SB_2 shared the identical SSRs in both organellar genomes. Therefore, Tsunewaki *et al.* (2019) concluded that *Ae. caudata* var. *polyathera* plasmon has not changed during its coexistence with the various foreign genomes described above in more than 60 generations.

Conclusion and future prospects

In this last challenging work presented above, Prof. Tsunewaki experimentally confirmed the genetic autonomy of plasmons in the *Triticum* and *Aegilops* complex, at least on a short evolutionary time scale (63 generations). Furthermore, based on his lifework on plasmon diversity and evolution, he attempted to estimate the extent to which plasmons have retained genetic autonomy in the evolution of this complex. Combining the results of the systematic studies, he classified 47 plasmons into 18 types along with 5 subtypes. In many cases, polyploid species, which differ in their genome constitution, exhibited identical or very similar plasmons to both each other and a diploid species with a related genome. Consequently, in these cases, their plasmon donors could be traced back to the diploid species, supporting the plasmon autonomy at the polyploid level. On the contrary, of the 18 major plasmon types and 5 subtypes, he detected 15 major types and 2 subtypes among diploid species. This result indicated that most of the plasmon diversity occurred at the diploid level. Based on these findings, he noted that the diploid species in the complex likely have had sufficient evolutionary time to genetically differentiate both the genome and the plasmon, while most polyploid species did not have enough time and thus retained their diploid-derived plasmons.

Recently, Noyszewski *et al.* (2014) reported the presence of heteroplasmic regions and significant differences between the mitochondrial genomes of the alloplasmic durum wheat line, (*Ae. longissima*)-*T. turgidum* subsp. *turgidum* conv. *durum* and its plasmon donor, *Ae. longissima*. In order to further verify the genetic autonomy at the whole nucleotide level in the lines produced by Prof. Tsunewaki, a whole genome sequencing analysis of the mitochondrial genomes of the (*caudata*)-Tve, (*caudata*^{Tve})-*caudata* var. *polyathera* and its plasmon do-

Fig. 3. Prof. Tsunewaki standing in front of the reconstructed *Ae. caudata* var. *polyathera* grown in his home garden. A photo was taken in May 2018.

nor, *Ae. caudata* var. *polyathera*, is underway.

Finally, I would like to mention that Prof. Tsunewaki completed most of his last work at the age of over 80 (Fig. 3). As a profound tribute to his passion and dedication to science, the alloplasmic lines (*caudata*)-Tve and (*caudata*^{Tve})-*caudata* var. *polyathera* are currently being maintained in our laboratory by repeated backcrossing and have reached SB_{69} and SB_6, respectively this year.

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