

SPECIFICATION

MARKER, METHOD AND KIT FOR OBSERVING EFFECT OF COMPOUND OR DRUG ON CELLS IN REAL TIME, AND USE THEREOF

FIELD OF THE INVENTION

The present invention belongs to the field of biology. More particularly, the present invention relates to a marker for observing an effect of a compound or a drug on cells in real time, and a method and a kit for observing an effect of a compound or a drug on cells in real time by using the marker, and use thereof.

BACKGROUND OF THE INVENTION

In the process of drug development and biological research, it is very important to study the effect of a molecule or a drug on cells. This is usually realized by observing the overall healthy condition of the cells, such as death or replication rate. The study on the effect of a molecule or a drug in this way only estimates a final result using the molecule or the drug, which may miss the effect of the molecule or the drug on the cells in a continuous process. In reality, a molecule or a drug is usually used for treating an aspect of cells or life, which is a continuous process before death or replication. Therefore, current methods for studying the effect of a molecule or a drug on cells are non-real-time, indirect or inaccurate. So far, there is no method for studying the real-time effect of a molecule or a drug on cells.

For any useful molecules, such as drugs, to be commercialized, side effects or toxicity must be tested. Generally, the tests can be conducted on animals, which can be very expensive and time consuming. In addition, people advocating animal rights also object to the drug tests on animals. Therefore, there is an urgent need for a sensitive method that can quickly provide a result without using animals to test the real-time effect.

SUMMARY OF THE INVENTION

Based on the demands and deficiencies of the prior art above, an objective of the present invention is to provide a marker for observing an effect of a compound or a drug on cells in real time; another objective of the present invention is to provide a method for observing an effect of a compound or a drug on cells in real time; another objective of the present invention is to provide a kit for observing an effect of a compound or a drug on cells in real time; and another objective of the present invention is to provide a method for observing a nonspecific toxicity of a compound or a drug in real time.

In order to achieve the objectives above, the following technical solutions are used in the present invention:

In one aspect, the present invention provides a marker for observing an effect of a

compound or a drug on cells in real time, wherein the marker is: 1) an amino acid sequence shown in SEQ ID NO: 1 and/or SEQ ID NO: 2; or

2) an amino acid sequence having a function for observing an effect of a compound or a drug on cells in real time and having at least more than 80%, preferably more than 85%, more preferably 90%, further preferably 95%, and most preferably 99% homology with the amino acid sequence shown in SEQ ID NO: 1 and/or SEQ ID NO: 2.

Preferably, the cells are selected from plant cells, yeasts, fungi, green algae or animal cells.

Further preferably, the effect is a nonspecific toxicity of the compound or the drug.

Further preferably, the observing an effect of a compound or a drug on cells in real time is to observe an movement of a VirE2-GFP on endoplasmic reticulum/actin network in real time.

Further preferably, the marker is a nucleotide encoding the marker.

In another aspect, the present invention provides a method for observing an effect of a compound or a drug on cells in real time, and the method comprises the following steps of:

1) introducing a marker for observing the effect of a compound or a drug on cells in real time or the marker above-mentioned to the cells, or enabling the cells to contain a marker for observing an effect of a compound or a drug on cells in real time or the marker above-mentioned;

2) adding the compound or the drug into the cells; and

3) observing a morphology, a movement track and/or a speed of the marker in the cells in real time.

Preferably, the cells are selected from plant cells, yeasts, fungi, green algae or animal cells.

More preferably, the morphology of the marker in the step 3) comprises a size, a shape, a movement mode and/or a position of a filamentous structure formed by the VirE2-GFP.

Further preferably, the observing an effect of a compound or a drug on cells in real time is to observe a movement of the VirE2-GFP on endoplasmic reticulum/actin network in real time.

Further preferably, the compound is a nucleotide and/or the drug is a small molecule drug.

More preferably, the compound or the drug is selected from a colchicine, a cytochalasin D, a Brefeldin A, an ML-7, an RFD-MBD, an MBD, an ER-mCherry, an ABD and a tail fragment of a myosin.

Further preferably, the effect is a nonspecific toxicity of the compound or the drug.

In another aspect, the present invention provides a kit for observing an effect of a compound or a drug on cells in real time, the cells are preferably selected from plant cells, yeasts, fungi, green algae or animal cells, and the kit contains a marker for observing an effect of a compound or a drug on cells in real time or the marker above-mentioned.

Preferably, the compound is a nucleotide and/or the drug is a small molecule drug.

More preferably, the compound or the drug is selected from a colchicine, a cytochalasin D, a Brefeldin A, an ML-7, an RFD-MBD, an MBD, an ER-mCherry, an ABD and a tail fragment of a myosin.

Further preferably, observing an effect of a compound or a drug on cells in real time is to observe a movement of a VirE2-GFP on endoplasmic reticulum/actin network in real time.

Further preferably, the effect is a nonspecific toxicity of the compound or the drug.

In another aspect, the present invention provides a method for observing a nonspecific toxicity of a compound or a drug in real time, and the method comprises the step of: using the marker above-mentioned and/or the kit above-mentioned.

According to the basic technical solutions of the present invention above, the concept of the present invention is further illustrated as follows.

The present invention has developed a method that can observe a real-time process of a whole movement of a protein (VirE2-GFP) generated in bacterial cells, delivered into a eukaryotic cytoplasm, and then moved to a cell nucleus. The whole process can be observed under a natural condition in real time. When a molecule or a drug is added to the test system, a real-time effect of the molecule or the drug on a movement process of cells or proteins can be directly observed.

Several compounds are tested in the present invention, some of which are drugs. The inventor finds that oral drugs on the market do not affect a real-time movement process of the protein VirE2-GFP, while toxic compounds can significantly interfere with the movement process of the protein VirE2-GFP.

Therefore, the present invention develops a technology that can directly observe a real-time effect of any compound or drug on cells, including a nonspecific toxicity. This may become an innovative drug research and development technology, and the technology can discover a nonspecific (or toxic) effect of a candidate drug at an early stage. In this way, drug development companies can evaluate a side effect and toxicity of any candidate drug as early as possible, thus saving time and money for drug development. The present invention also provides a platform for the public to directly and quickly evaluate the toxicity and non-specificity of any drug. This also helps to build confidence in drug testing for the public.

Method

The present invention develops a cell-based system which includes both bacterial cells and eukaryotic cells. A split-GFP approach is adopted to visualize an *Agrobacterium*-produced protein VirE2, which can be naturally delivered by *Agrobacterium* into eukaryotic cells. To visualize VirE2 delivered into eukaryotic cells, the small GFP fragment (GFP11) is inserted into VirE2 at a permissive site to create VirE2-GFP11 fusion, which is expressed in *A. tumefaciens*; and the large fragment (GFP1-10) is expressed in recipient cells. Upon delivery of VirE2-GFP11 into the recipient cells, GFP fluorescence signals are visualized. VirE2-GFP11 is functional like VirE2; the GFP fusion movement could indicate the

trafficking of *Agrobacterium*-delivered VirE2.

This system can be used to visualize the entire movement of a protein produced in a bacterial cell, delivered onto a eukaryotic cytoplasm, and then moved into the nucleus. The entire process can be visualized in real time in natural setting. When a compound is added into the testing system, the effect of the compound on any of the protein trafficking process can be visualized directly.

VirE2 has the ability to aggregate to filamentous structures, which can move smoothly inside the cells. They can be easily visualized and documented. Any effect on the cells would affect the sizes, shapes, movement patterns and locations of the VirE2 filaments. This makes the test particularly sensitive.

Result

Several compounds are tested in the present invention, some of which are drugs. The inventor finds that oral drugs on the market are less toxic and do not affect a real-time movement process of a protein, while toxic compounds can significantly interfere with the movement process of a protein.

The test system covers all cell positions and all important biochemical processes. The test is very sensitive and can detect any potential nonspecific reaction. The test is very quick and can be completed in two days.

Analysis

Therefore, the present invention develops a technology that can directly visualize the non-specific effects of any drugs in real time. This can potentially revolutionize drug discovery process by revealing the non-specific (or toxic) effects of drug candidates at early stage. In this way, drug development companies can assess the side-effects and toxicity for any drug candidates as early as possible to save the time and money for drug development. It also provides a platform for the public to assess the toxicity and non-specificity of any drug in a direct and quick manner. This is certainly useful for the public to build up the confidence for drug testing.

Conclusion

The present invention develops a technology that makes it visible to test the non-specific or toxic effects of any drugs. The test can provide a mechanistic view of the nonspecific or toxic effect directly. This can quickly test whether drug candidates have potential non-specific or toxic effect at the early stage so that time and money can be saved for drug discovery.

The safety of a drug can be evaluated in a visual and understandable manner so that the public confidence on the drug can be improved. The testing system can help biological researchers to conduct mechanistic studies on any molecule. The technology can be used to develop an un-biased service center to test the potential non-specific or toxic effects of drug candidates and existing drugs on the markets.

In addition, the present invention can also be used for observing an effect of a compound or

a drug on cells in real time, thus helping basic research.

BRIEF DESCRIPTION OF THE DRAWINGS

The embodiments of the present invention are described in detail hereinafter with reference to the drawings, wherein:

Fig. 1 illustrates *Agrobacterium*-delivered VirE2 trafficking on a cellular structure and entering the nucleus;

Fig. 2 illustrates *Agrobacterium*-delivered VirE2 co-localizing with and trafficking on the ER network;

Fig. 3 illustrates *Agrobacterium*-delivered VirE2 co-localizing and trafficking on F-actin filaments;

Fig. 4 illustrates the effects of ML-7 and myosin-tail overexpression on VirE2 trafficking;

Fig. 5 illustrates the effects of chemicals on respective cellular structures;

Fig. 6 illustrates that *Agrobacterium*-delivered VirE2 is present on the cytosolic side of ER;

Fig. 7 illustrates the effects of myosin-tail overexpressions on VirE2 trafficking;

Fig. 8 illustrates a time-lapse imaging of a NLS1-mutant VirE2 moving on an endoplasmic reticulum filament;

Fig. 9 illustrates the effects of ML-7 treatment on root tumorigenesis assay;

Fig. 10 illustrates the effects of RNAi silencing of XI-2 and XI-K on tumor formation; and

Fig. 11 illustrates an effect of a drug on the VirE2 movement.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Scheme

As a soil-borne phytopathogen, *A. tumefaciens* is capable of inter-kingdom transfer of genetic materials. It can deliver single-stranded (ss) DNA (T-DNA) through a VirB/D4 type IV secretion system (T4SS) into various recipient cells. Although plant species are the natural hosts for this T-DNA transfer, other eukaryotic species can nevertheless be transformed under laboratory conditions, including yeast, fungal and algal cells, in the presence of plant wound signal compounds, such as acetosyringone. The ability of *Agrobacterium* to genetically modify recipient cells is widely used for plant and fungal transformation.

The T4SS are found both in Gram-negative and Gram-positive bacteria; they secrete proteins and nucleoprotein complexes into recipient cells. *A. tumefaciens* T4SS apparatus is encoded by the *vir* genes on the Ti plasmid; it comprises 12 types of toxic proteins (*virB1* to *virB11* and *virD4*). During the transfer process, T-DNA is nicked and processed from T-region on the Ti-plasmid by VirD1-VirD2 endonuclease inside the bacteria. The nucleoprotein complex formed by VirD2 and T-DNA is then translocated through the T4SS

to recipient cells, along with other virulence proteins, like VirD5, VirE2, VirE3 and VirF. In the recipient cell, these translocated proteins act as virulence effectors to facilitate the transformation process.

VirE2 is the most abundant among the bacterium-encoded Vir proteins. VirE2 can bind to T-DNA in a cooperative manner; it plays the critical role of protecting T-DNA from nucleolytic degradation during cytoplasmic trafficking inside the host cells. This occurs as VirE2 has the natural ability to bind to ss DNA and to self-aggregate to form solenoid super structures. VirE2 contains nuclear localization signals (NLS) that facilitate the nuclear import of VirE2 and potentially the T-DNA. There is evidence to suggest that VirE2-T-DNA interaction also plays a role in targeting the T-DNA into the nucleus, independent of the nuclear targeting activity of VirD2 which also contains the NLS. Although ectopic expression of VirE2 showed a predominant cytoplasmic localization of VirE2 in various types of plant cells, we found that using a split-GFP approach, a significant amount of *Agrobacterium*-delivered VirE2 was localized inside the plant nuclei under natural infection conditions. Indeed it is of significance to study the trafficking process of VirE2 in the cytoplasm of host cells in order to understand the transformation mechanism.

As VirE2 aggregates as the solenoid structure, its large size would strongly restrict it from reaching the host nuclei through a dense structure of cytoplasm by Brownian diffusion; there should be an active mechanism for VirE2 trafficking inside the plant cells. Previous studies showed that the interaction of VirE2 with a transcription factor VIP1 may facilitate VirE2 nuclear targeting by abusing the MAPK-targeted VIP1 defense signaling pathway. However, the role of VIP1 in *Agrobacterium*-mediated transformation is debatable. Nevertheless, it is not clear how any of the bacterial effectors and their host partners is trafficked inside host cells to facilitate the transformation.

An *in vitro* study showed that the presence of “animalized” VirE2, in which the VirE2 NLS was modified to become a bipartite NLS similar to nucleoplasmin, invoked active transport along microtubules in a cell-free *Xenopus* egg extract. Since microtubules are projected radially from the centrosome in animal cells, this trafficking mechanism would effectively transport nuclear-targeted cargo close to the nuclear envelope for import. Ectopically expressed VirE2 in yeast was also reported to co-localize and physically interact with microtubules. These lines of evidence suggest the involvement of microtubules in VirE2 trafficking. However, unlike animals and fungi, flowering plants lack retrograde transporter-dyneins. Moreover, plant microtubules lack conspicuous organizing centers; their arrangements are fundamentally different from the animal, fungal and protistan counterparts; microtubules may not be ideal for trafficking towards plant nuclei. Thus, it is not clear whether plant microtubules play any role in VirE2 trafficking towards the nucleus in a natural setting.

So far no natural system has been used to study the trafficking of VirE2 or any of the T-complex components; and conclusions based on *in vitro* experiments also have great limitations. Consequently, it is still unknown what kind of host network drives the trafficking of T-complex inside plant cells.

The present invention develops a split-GFP-based method that can directly detect the *Agrobacterium*-delivered VirE2 inside plant cells. This split-GFP approach enabled us to visualize VirE2 trafficking in the recipient cells in real-time in a natural setting. The inventor found that *Agrobacterium* transformed plant cells at a high efficiency up to 100%. The experimental results of the present invention showed that VirE2 was trafficked through myosin-powered ER/actin network inside plant cells. Since actin network is well conserved, our data suggest that *Agrobacterium* hijacks host fundamental infrastructure to achieve both the high efficiency and the wide host range for the transformation.

Result

Agrobacterium-delivered VirE2 moved on a strand-like cellular structure

The inventor has successfully observed that *Agrobacterium*-delivered VirE2 inside plant host cells using the split green fluorescence protein (split-GFP) method. According to the present invention, *A. tumefaciens* cells (EHA105*virE2::GFP11*), encoding VirE2-GFP11 fusion, are infiltrated into the leaf tissues of tobacco plants (Nb308A), which constitutively express GFP1-10 and the free DsRed that indicates the cellular structures and the nucleus. Upon delivery into plant cells by the bacterium, VirE2-GFP11 complemented GFP1-10, the resulting VirE2-GFP_{comp} signals were found inside plant cells. At 2 days after agroinfiltration, VirE2-GFP_{comp} aggregates started to appear, as VirE2 can aggregate to form filamentous structures.

As shown in Fig. 1a, VirE2-GFP_{comp} signals moved on a strand-like cellular structure illustrated by the free DsRed. More strikingly, direct entry of VirE2 into the nucleus was visualized. To ensure that this observation was not a false co-localization due to an axial projection, a 3D opacity view of the nucleus was generated based on the same image data set; 3D rotation showed that the optical slices and VirE2-GFP_{comp} signals were within the nucleus. This confirmed that the VirE2-GFP_{comp} signal entered the nucleus. VirE2 moved at a speed of 1.1 $\mu\text{m}/\text{sec}$ along the linear track, but it slowed down around the nucleus area. The average velocity was 0.434 $\mu\text{m}/\text{sec}$ for this trafficking event observed. Generally speaking, VirE2 moved faster along a linear track although the velocities varied on different linear tracks; it moved slower along curved tracks.

VirE2 trafficking was sensitive to cytochalasin D and Brefeldin A

Next, in order to study the nature of cellular structure that facilitated the movement of *Agrobacterium*-delivered VirE2 inside plant cells. Transgenic plants (Nb308A) expressing GFP1-10 and free DsRed were treated with chemicals known to disrupt cellular structures. As shown in Fig. 1b-d, cytochalasin D (CytoD) and brefeldin A (BFA) severely affected the VirE2 trafficking, while colchicine (Colc) only exhibited a minor effect. The inventor monitored over 20 independent events of VirE2 movements for each treatment. The inventor found that CytoD and BFA treatment reduced the average velocities of VirE2 movement to less than 20%, while Colc treatment showed over 60% of the average velocity of the control (Fig. 1c).

CytoD is a potent inhibitor of actin polymerization, and BFA inhibits protein transport from endoplasmic reticulum (ER) to Golgi apparatus by dilating the ER, while Colc inhibits

microtubule polymerization. The inventor observed the effects of these inhibitors on the corresponding cellular structures in tobacco leaves under test conditions. Colc, BFA and CytoD indeed disrupted the microtubule, ER and actin structures, respectively. Therefore, the invention hypothesized that VirE2 trafficking was probably facilitated by ER/actin structures.

VirE2 movement was associated with endoplasmic reticulum.

In order to determine if VirE2 movement is associated with the endoplasmic reticulum, the present invention uses an ER-mCherry construct containing an ER targeting sequence at the N-terminus and the tetrapeptide retrieval signal HDEL at the C-terminus. Both GFP1-10 and ER-mCherry constructs were then introduced into a T-DNA harbored on a binary plasmid to generate pQH308ER. The plasmid was introduced into EHA105*virE2::GFP11* expressing VirE2-GFP11 that is functional like wild-type VirE2.

The inventor infiltrated EHA105*virE2::GFP11*(pQH308ER) into *N. benthamiana* leaves and then observed *Agrobacterium*-delivered VirE2-GFP_{comp} signals. As shown in Fig. 2a, VirE2 aggregates appeared as dots or filaments inside tobacco cells. Both forms were co-localized with inter-connected ER tubules illustrated by ER-mCherry. The VirE2 filaments matched with the ER strands in an imperfect manner (lower panel). It is not clear whether the imperfect matching was real or due to the gap time required to detect the two different moving colors. Time-lapse imaging showed that VirE2 aggregates moved along the ER strands (Fig. 2b); the average velocity was 0.502 $\mu\text{m}/\text{sec}$ during this linear movement, which is consistent with our earlier observations.

VirE2 exists on a cytosol side of the endoplasmic reticulum in the plant cells.

The inventor determined if VirE2 was present on the cytosolic or luminal side, as ER is an interconnected network of flattened, membrane-enclosed sacs or tubes known as cisternae and the ER membranes compartmentalize into ER lumen and cytosol. The inventor generated an ER-GFP1-10 construct containing an ER targeting sequence at the N-terminus of GFP1-10 and an ER retention signal HDEL at the C-terminus. The inventor also generated a two-tandem GFP11 (2 $\times$ GFP11) and its ER-localizing construct (ER-2 $\times$ GFP11) containing an ER targeting sequence at the N-terminus and an ER retention signal HDEL at the C-terminus.

These constructs were expressed in wild type *N. benthamiana* leaves by agro-infiltration. GFP_{comp} signals were detected when ER-GFP-1-10 was co-expressed with ER-2 $\times$ GFP11, but not with 2 $\times$ GFP11 (Fig. 6a-b). This demonstrated that the ER-GFP1-10 construct was indeed localized inside ER lumen. EHA105*virE2::GFP11* containing VirE2-GFP11 was infiltrated into *N. benthamiana* leaves which transiently expressed either GFP1-10 or ER-GFP1-10. As shown in Fig. 6c, *Agrobacterium*-delivered VirE2-GFP11 complemented GFP1-10, but not ER-GFP1-10. This demonstrated that *Agrobacterium*-delivered VirE2 was on the cytosolic side of ER after delivery into plant cytoplasm.

VirE2 moves along a F-actin filament.

Since the CytoD can stop the VirE2 movement (Figs. 1b-d), and CytoD can be bound to the F-actin and prevent the monomer polymerization of F-actin, the present invention

attempts to determine whether the VirE2 movement is associated with the F-actin filament.

In order to observe the F-actin and VirE2 simultaneously, the inventor injects and infiltrates the *A. tumefaciens* strain EHA105*virE2::GFP11* into the tobacco plant (Nb307A) expressing the GFP1-10. Meanwhile, the strain carries a binary plasmid encoding a F-actin marker tdTomato-ABD2 for expression in the tobacco. As shown in Fig. 3, the VirE2-GFPcomp signal overlaps with a position of the F-actin filament. The time-lapse imaging shows that the VirE2 moves along the F-actin filament.

VirE2 movement depends on Mmyosin.

Since the endoplasmic reticulum/F-actin/myosin can perform a three-way interaction, the inventor then decides to examine whether the myosin plays a role in the VirE2 movement in the plant cells. Firstly, the present invention uses a selective myosin light chain kinase inhibitor ML-7 to inhibit an activity of a plant myosin. As shown in Fig. 4, ML-7 treatment obviously stops the VirE2 movement. Compared with a control group, an average rate of the movement of the VirE2 decreases by 95%.

Next, the inventor uses a dominant-negative approach to identify the specific myosin responsible for VirE2 movement. Several dominant negative mutants of plant myosin genes were overexpressed during *Agrobacterium*-mediated delivery of VirE2, since headless myosin tails could interrupt myosin activity in a dominant negative manner. *A. tumefaciens* cells containing T-DNA harboring the tail constructs were co-infiltrated with EHA105*virE2::GFP11* into tobacco plants (Nb308A) expressing GFP1-10 and the free DsRed; the myosin tail expression would take place later than VirE2 delivery so that the myosin mutant constructs would not affect the VirE2 delivery. Among the myosin mutants tested, only XI-K tail remarkably arrested the VirE2 trafficking (Fig. 4c and Fig. 7c); the average velocity was reduced to 10% as compared to the control (Fig. 4d). In contrast, overexpression of XI-2 and other myosin tails exhibited only a minor or insignificant effect (Fig. 4c and Fig. 7c). These data suggest that myosins provide the driving force for VirE2 movement and myosin XI-K was the most important contributor.

It is of particular interest to determine if the VirE2 movement inside the cell is dependent upon the NLS. Since a mutation at NLS1 of VirE2 rendered VirE2 non-functional, any ER marker construct would not be expressed transiently using the VirE2 NLS1 mutant strain. However, introducing a second strain to deliver functional VirE2 would inevitably generate chimeric VirE2 aggregates which could interfere with the behavior of VirE2 Δ NLS1. Therefore, the present invention establishes transgenic tobacco Nb308ER, which constitutively express GFP1-10 and ER-mCherry.

EHA105*virE2 Δ NLS1::GFP11* was infiltrated into the epidermal cells of Nb308ER. As shown in Fig. 8, VirE2 Δ NLS1 was co-localized with ER strands and moved along the ER strands. This demonstrated that VirE2 movement inside the cytoplasm was independent of NLS, although NLS is required for nuclear targeting.

Myosin XI-K is required for *A. tumefaciens*-mediated transformation.

The inventor wants to determine if the VirE2 movement observed during our studies is directly relevant to *Agrobacterium*-mediated transformation. The present invention tested

the effect of a selective myosin light chain kinase inhibitor ML-7 on *Arabidopsis* root transformation. Root segments were inoculated with a tumor inducing strain A348 in the presence of 10 μ M ML-7. As shown in Fig. 9, ML-7 significantly reduced the transformation efficiency. The present invention tested the toxicity of ML-7 on either the root or *Agrobacterium* growth at 10 μ M ML-7. The root segments were exposed to 10 μ M of ML-7 for 2 days, which was the time span for the bacterium-*Arabidopsis* co-cultivation. As shown in Fig. 9c, ML-7 did not affect the growth of root segments in the presence of hormones (auxin and cytokinin); ML-7 did not inhibit *Agrobacterium* growth either (Fig. S5d). These suggest that inhibition of myosin activity could reduce the transformation efficiency.

To confirm the specific effect of myosin inhibition on transformation, the inventor uses RNAi constructs containing partial sequence of XI-2 and XI-K (48) to silence the corresponding genes. The RNAi constructs used for the studies could generate specific effects but not off-target effects. As shown in Fig. 10, silencing of XI-K evidently attenuated tumor formation. The data clearly indicated that XI-K affected the VirE2 movement and consequently affected *Agrobacterium*-mediated transformation.

Toxicity determination of drug

The present invention tests several drugs and finds that drug Ketoconazole on the market does not affect a real-time movement process of a protein VirE2-GFP and is considered to be less toxic; while Econazole significantly affects the real-time movement process of the protein VirE2-GFP and is considered to be highly toxic (Fig. 11).

Discussion

A. tumefaciens has an exceptional ability to transform a wide range of host plants in nature and various recipient cells under laboratory conditions. In addition, the bacterium can achieve a high efficiency of transformation up to 100% of the plant cells in contact with the bacteria. The high efficiency enabled us to dissect the movement of *Agrobacterium*-delivered VirE2 inside plant cells. As the present invention shows here, the bacterium hijacks conserved host network to move virulence factor VirE2 towards the nucleus. This might be important for *Agrobacterium* to achieve both a wide host range and a high efficiency.

In order to transform plant cells, *Agrobacterium* must be able to deliver its virulence factors into host cells efficiently. These exogenous factors must be able to move towards appropriate locations to exercise their functions inside host cells. It is indeed a challenge to study how these proteins are trafficked through the cytoplasm and reach the nucleus, as this is a complex process and any disturbance to the process in an *in vitro* system might generate artefacts.

The present invention adopted a split-GFP approach to visualize VirE2 in a natural setting, which made it possible to monitor intracellular trafficking of the virulence effector VirE2 in real time. The studies showed that *Agrobacterium*-delivered VirE2 were trafficked *via* endoplasmic reticulum (ER) and F-actin network; this process was powered by myosin XI-K in particular. It is known that ER, actin filaments and myosins can interact with each

other to form a network. The data of present invention showed that exogenously produced and then delivered VirE2 protein complex could use endogenous host ER/actin network for its trafficking inside host cells.

VirE2 is critical for the fate of T-DNA in many ways. Therefore, it is of particular significance to study how VirE2 is trafficked through the cytoplasm and reaches the nucleus. VirE2 contains two bipartite NLS signals, which are present on the exterior side of the solenoidal structure. This kind of structural arrangement may make the NLS signals available to interact with other host factors for VirE2 trafficking.

Indeed, VirE2 was shown to interact with *Arabidopsis* importin α isoform IMPa-4 when both proteins were overexpressed; VirE2 localized in the plant nucleus. When the NLS of VirE2 was mutated to be recognizable in animal cells, the “animalized” VirE2 was found to migrate along microtubules in cell-free *Xenopus* oocyte extracts, and dynein motors were important for this migration. However, no plant dyneins have been found. It remains unknown whether VirE2 moves along microtubules in plant cells. The “animalized” VirE2 trafficking in an animal cell context therefore might not necessarily represent the mode of VirE2 trafficking inside plant cells. In fact, disruption of microtubules by colchicine did not affect the VirE2 trafficking significantly (Fig. 1d). These suggest that VirE2 uses a trafficking system other than microtubules for its trafficking inside plant cells.

In an *in vivo* trafficking system, there was no disturbance to the cellular infrastructures; the studies revealed myosin-dependent trafficking of VirE2 *in planta* in a natural setting. Presumably, VirE2-associated T-complex may also use the same trafficking mode, as VirE2 can coat T-complex on the surface. Therefore, this study may provide a plausible explanation to the tremendous difference in transformation efficiency between yeast and plant recipients (0.2% in *S. cerevisiae* vs 100.0% in *N. benthamiana*), while the efficiency of protein delivery is comparable (50.9% in *S. cerevisiae* vs 100.0% in *N. benthamiana*). VirE2 trafficking may require plant-specific myosin XI family, XI-K in particular. The budding yeast *Saccharomyces cerevisiae*, on the other hand, lacks myosin XI-K. This would render VirE2 immobile in the yeast cells; thus the transformation efficiency is strongly attenuated as reported previously.

Myosins are the important motor proteins that move on actin filaments in eukaryotic cells. In plant cells, myosins can be categorized into three main classes, myosin VIII, XI and XIII. Myosin XI family members are evolutionarily related to the animal myosin V family, and are involved in the cytoplasmic streaming, ER motility, and trafficking of organelles and vesicles. The myosin XI family comprises fast motor members that can traffic at several micrometers per second, which surpasses its myosin V family counterparts by over ten folds. Despite the conformational similarity with myosin V, myosin XI has a plant specific binding mechanism and thus recognizes cargos that are distinct from myosin V. Our studies demonstrated that myosin XI-K played a much more critical role in VirE2 trafficking than XI-2. Both XI-K and XI-2 are highly expressed inside plant cells. However, myosin XI-K is the primary contributor to ER streaming as compared to XI-2.

The present invention assumes that that *Agrobacterium* has evolved to enable VirE2 to take the free-ride of a fundamental process: ER streaming, which is part of cytoplasmic

streaming. *Agrobacterium*-delivered VirE2 was associated with ER (Fig. 2b), presumably because the high affinity of VirE2 to membranes enabled VirE2 to associate with ER, which has extensive membrane structures. Of course, it is also possible that an unknown factor(s) is responsible for VirE2-ER association. VirE2-associated ER is then driven primarily by ER-associated myosin XI-K. Myosin-associated ER can move along actin filaments. Therefore, *Agrobacterium*-delivered VirE2 is trafficked through plant cells *via* myosin-powered ER/actin network, because of the dynamic three-way interactions between ER, F-actin and myosin. At this stage, it is not clear which part of VirE2 is required for VirE2 movement. This remains to be solved in the future.

The endoplasmic reticulum stretches through the entire cytoplasm and continues with outer membrane of nucleus, which would provide VirE2 a convenient path to reach the nucleus. Cytosolic facing of VirE2 on the ER would presumably make the opening of nuclear pore complex accessible for nuclear import of VirE2 that contains the NLS. Moreover, the association of VirE2 with ER also suggests that VirE2 may interact with other factors during the trafficking processes. Indeed, a SNARE-like protein was found to have a strong interaction with VirE2. This suggests that vesicular budding or fusion processes may be involved in VirE2 trafficking inside the cytoplasm. Currently, it is still not clear whether any of other bacterial virulence proteins delivered by *Agrobacterium* would be trafficked along with VirE2. It remains to be established how other bacterial virulence proteins are trafficked inside host cells upon the delivery. These issues should be examined in the future studies, which could lead to important findings that may provide new insight into the transformation process.

Optimum mode for implementing invention

The present invention is further described by specific embodiments with reference to the drawings, but it shall be understood that these embodiments are only for the object of more detailed description and shall not be construed as limiting the present invention in any form.

This section gives a general description to the materials and experimental methods used in experiments of the present invention. Although many materials and operation methods used to achieve the objective of the present invention are well known in the art, the present invention is still described here in as much detail as possible. It is clear to those skilled in the art that the materials and operation methods used in the present invention are well known in the art in the context, unless otherwise specified.

First of all, the materials and experimental methods used in various embodiment are uniformly described as follows.

Strains, plasmids, primers and growth conditions

The strains and the plasmids used in the experiment are listed in Table 1.

Table 1 Strains and Plasmids Used in Experiment

Strain and plasmid	Correlation characteristic
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<i>A. tumefaciens</i>	
EHA105	A C58 strain contains a pTiBo542 plasmid, contains a <i>vir</i> gene, but does not contain a T-DNA
EHA105 <i>virE2::GFP11</i>	An EHA105 derivative strain, comprising 162 bp after ATG with a GFP11 coding sequence inserted into a <i>virE2</i> gene on a pTiBo542
EHA105 <i>virE2::GFP11nlsI</i>	An EHA105 <i>virE2::GFP11</i> derivative strain, the first nuclear targeting signal sequence is replaced with alanine from 221KLR...KYGRR237
A348	A136 (pTiA6NC) (Octopine-type)
Plasmid	
pQH308A	The GFP1-10 coding sequence is inserted into a pDs-Lox and replaces a Bar in the pDs-Lox. An obtained Pmas:GFP1-10:Tnos component is amplified by a PCR and inserted into a <i>ClaI</i> - <i>HindIII</i> site on a pBI121; a DsRed ORF is inserted into an <i>XbaI</i> - <i>BamHI</i> site on the pBI121 and under a 35S promoter.
pER-rk	A binary plasmid encodes a mCherry sequence located on an ER.
pQH308ER	An ER-mCherry sequence cloned from a pER-rk is replaced with a DsRed coding sequence on a pQH308A.
pQH307A	The DsRed coding sequence on the pQH308A is deleted.
pCB302	Mini binary plasmid with the 35S promoter
pCB302-XIK-IQC	A pCB302 plasmid expresses a fragment of a tail end of a myosin XI-K from Ala731.
pCB302-XI2-IQC	pCB302 plasmid expresses a fragment of a tail end of a myosin XI-2 from Val735.
pCB302-XIF-IQC	pCB302 plasmid expresses a fragment of a tail end of a myosin XI-F from Ile740.
pCB302-VIII1-IQC	pCB302 plasmid expresses a fragment of a tail end of a myosin VIII1 from Thr827.
pCB302-VIII2-IQC	pCB302 plasmid expresses a fragment of a tail end of a myosin VIII2 from Glu864.
pCB302-VIIIB-IQC	pCB302 plasmid expresses a fragment of a tail end of a myosin VIIIB from Val831.
pCB302-dsXIK	pCB302 plasmid expresses an RNAi component, covering a nucleotide sequence interval of open reading frames 3,153–3,357 of the myosin XI-K
pCB302-dsXI2	pCB302 plasmid expresses the RNAi component, covering a nucleotide sequence interval of open reading frames

	3,146–3,357 of the myosin XI-2
pB5tdGW-ABD2	binary plasmid encodes a tdTomato-fusion actin.
pH7WGR2-RFP-MBD	The binary plasmid encodes an RFP-fusion microtubule binding domain.
pBI121	The binary plasmid has the 35S promoter and a Gus-A coding sequence.
pQH121	The Gus-A coding sequence in the pBI121 is replaced by a multiple cloning site.
pQH121-ER-GFP1-10	A pQH121 encodes the GFP1-10 sequence, and is fused with an ER localization signal at the N end and is fused with an HDEL ER-reservation signal at the C terminus.
pQH121-2×GFP11	The pQH121 encodes two GFP11 in series.
pQH121-ER-2×GFP11	The pQH121 encodes two GFP11 in series, and is fused with the ER localization signal at the N end and is fused with the HDEL ER-reservation signal at the C terminus.

A. tumefaciens (*agrobacterium tumefaciens*) grows in a MG/L medium and an induction medium (IBPO4) at 28°C. An *Escherichia coli* (*E. coli*) DH5 α strain is used for plasmid construction and grows in a LB medium at 37°C.

Plasmid construction and production of transgenic tobacco plant (*N. benthamiana*)

Binary plasmids harboring a T-DNA region that encodes either a GFP1-10 expression cassette or a GFP1-10 and ER-mCherry dual cassettes were constructed and then used to generate transgenic *N. benthamiana* lines. Plasmid pQH308A(28) was digested with *Xba*I and *Sac*I to remove the coding sequence of DsRed. The linearized plasmid was then re-ligated after the overhanging sticky ends were blunted by Klenow fragment, resulting in pQH307A. The coding sequence of ER-mCherry from pER-rk (44) was cloned into the *Xba*I-*Sac*I site of pQH308A to replace DsRed, resulting in pQH308ER.

Agrobacterium-mediated transformation of *N. benthamiana* plants was performed using leaf sections. Transgenic calli were generated on Murashige and Skoog (MS) media (64) supplemented with 100 mg L⁻¹ kanamycin, 2 mg L⁻¹ 6-BA and 0.2 mg L⁻¹ NAA. Transgenic tobacco plantlets were obtained by transferring the calli with shoots into 1/2 MS plates supplemented with 0.1 mg L⁻¹ IBA. Transgenic lines were then named as Nb307A and Nb308ER to reflect the corresponding binary plasmids pQH307A and pQH308ER.

Virulence assays

Arabidopsis thaliana seeds were surface-sterilized with 0.5% NaClO solution and placed onto solidified 1/2× MS medium supplemented with 1% sucrose and 0.5 g L⁻¹ MES, pH 5.8. The plates were then incubated under a 16 hour photoperiod at 25 °C for 10 to 12 days. Roots from individual seedlings were cut into 3-5 mm segments and re-suspended in 1 ml fresh 1/2× MS medium containing *A. tumefaciens* cells at a concentration of 5 × 10⁸ cell/ml unless specified otherwise. The mixtures were spread onto a solidified 1/2× MS plate and subsequently incubated at 25 °C for 2 days. The root segments were aligned onto 1/2× MS

medium plates containing 100 $\mu\text{g mL}^{-1}$ cefotaxime and kept at 25 °C for 4 weeks before photographing.

Leaves of *N. benthamiana* were surface sterilized with 0.5% NaClO and punctured into discs. The leaf discs were resuspended into 1/2×MS medium containing *A. tumefaciens* cells at a concentration of 1×10^8 cell/ml unless specified otherwise. The leaf discs were aligned onto a 1/2× MS plate and subsequently incubated at 25 °C for 2 days. The leaf discs were then transferred onto another 1/2× MS plate supplemented with 100 $\mu\text{g mL}^{-1}$ cefotaxime and kept at 25 °C for 2 weeks before imaging.

Agroinfiltration

To visualize *Agrobacterium*-delivered VirE2, agroinfiltration was performed as described previously. Briefly, the bacteria were grown overnight; the cultures were diluted 50 times in MG/L and grown for 6 h. The bacteria were collected and re-suspended in infiltration buffer (10 mM MgCl_2 , 10 mM MES, pH5.5) to $\text{OD}_{600}=1.0$. The bacterial suspension was infiltrated using a syringe to the underside of fully expanded *N. benthamiana* leaves. The infiltrated plant was placed at 22°C in a photoperiod of 16 h light/8 h dark. In order to observe a real-time effect of a compound or a drug on cells in real time, the inventor injected and infiltrated the compound or the drug into a leaf injected and infiltrated with *A. tumefaciens* EHA105virE2::GFP11 cell at an appropriate concentration, and observed with a UPLSAPO ×60 N.A. 1.20 water immersion lens under a confocal microscope after six hours.

Detection of mRNA by qRT-PCR method

To quantify the mRNA level in RNAi-silenced plants, total RNA from plants for each treatment was extracted and reverse transcribed by iScript cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR was performed in triplicates with KAPA SYBRs on CFX384 Real-Time PCR system (Bio-Rad) by using the actin gene as an internal control (5'-CTTGAAACAGCAAAGACCAGC-3' and 5'-GGAATCTCTCAGCACCAATGG-3'). Gene specific primers for qRT-PCR are as follows: 5'-TCGTTTCGGTAAGTTTGTGG-3' and 5'-CATTGCCCTTCTTGTAGCC-3' for *N. benthamiana* myosin XI-2 gene (accession number DQ875135), 5'-GAATCAGTGAGGAAGAGCAGG-3' and 5'-CCGTCATATTGAGATGAAATCG-3' for *N. benthamiana* myosin XI-K gene (accession number DQ875137).

Confocal microscopy

A PerkinElmer UltraView Vox Spinning Disk system with EM-CCD cameras was used for confocal microscopy. To observe leaf epidermis, agroinfiltrated leaf tissues were detached from *N. benthamiana* plants and put in 2% low-melting agarose gel on a glass slide with a coverslip. All images were taken in multiple focal planes (Z-stacks), and were processed to show the extended focus image or 3D opacity view by Volocity® 3D Image Analysis Software 6.2.1.

Embodiment 1: *Agrobacterium*-delivered VirE2 trafficking on a cellular structure and entering the nucleus.

Fig. 1 illustrated *Agrobacterium*-delivered VirE2 trafficking on a cellular structure and entering the nucleus. *A. tumefaciens* EHA105virE2::GFP11 cells were infiltrated into transgenic *N. benthamiana* (Nb308A) leaves expressing both GFP1-10 and DsRed. The leaf epidermal cells were observed at 2 d post agroinfiltration under confocal microscope with UPLSAPO $\times 60$ N.A. 1.20 water immersion objective. Red, free DsRed; Green, VirE2-GFP_{comp}. (a) Time-lapse images of VirE2 aggregates trafficking along a linear cellular structure and entering the nucleus. Relative time is shown at the top right. Scale bar represents 20 μ m. (b) Effects of chemical treatments on VirE2 trafficking. Chemicals were infiltrated into leaf samples 6 h before observation. Control, 0.5% DMSO. Colc, colchicine 500 μ M. CytoD, cytochalasin D 20 μ M. BFA, brefeldin A 100 μ g/mL. Scale bar represents 20 μ m. (c) Mean velocities of VirE2 aggregate movement after chemical treatments. Data analyzed with ANOVA and Tukey test, $p < 0.05$. (d) Movement tracking of 20 individual VirE2 aggregates plotted to a common origin for each treatment.

Embodiment 2: *Agrobacterium*-delivered VirE2 co-localizing with and trafficking on the ER network

Fig. 2 illustrated *Agrobacterium*-delivered VirE2 co-localizing with and trafficking on the ER network. *A. tumefaciens* EHA105virE2::GFP11 cells harboring a binary plasmid pQH308ER, which encodes an ER-mCherry marker driven by 35S promoter and GFP1-10 driven by *mas* promoter, were infiltrated into wild type *N. benthamiana* leaves. The leaf epidermal cells were observed at 2 d post agroinfiltration under confocal microscope with UPLSAPO $\times 60$ N.A. 1.20 water immersion objective. Red, ER-mCherry; Green, VirE2-GFP_{comp}. (a) VirE2 aggregates co-localizing with inter-connected ER tubules. Scale bar represents 10 μ m. (b) Time-lapse images of VirE2 aggregates trafficking on an ER strand. Relative time is shown at the top right. Scale bar represents 20 μ m.

Embodiment 3: *Agrobacterium*-delivered VirE2 co-localizing and trafficking on F-actin filaments

Fig. 3 illustrated that *Agrobacterium*-delivered VirE2 co-localizing and trafficking on F-actin filaments. *A. tumefaciens* EHA105virE2::GFP11 cells bearing a binary plasmid pB5tdGW-ABD2, which encodes an actin marker tdTomato-ABD2 driven by 35S promoter, were infiltrated into transgenic *N. benthamiana* Nb307A which constitutively expressing GFP1-10. The leaf epidermal cells were observed at 2 d post agroinfiltration under confocal microscope with UPLSAPO $\times 60$ N.A. 1.20 water immersion objective. Red, F-actin; Green, VirE2-GFP_{comp}. (a) VirE2 aggregates co-localizing with F-actin filaments. Scale bar represents 20 μ m. (b) Time-lapse images of VirE2 aggregates trafficking on F-actin filaments. Relative time is shown at top right. Scale bar represents 10 μ m.

Embodiment 4: Effects of ML-7 and myosin-tail overexpression on VirE2 trafficking

Fig. 4 illustrated effects of ML-7 and myosin-tail overexpression on VirE2 trafficking. (a) Effect of ML-7, a myosin light chain kinase inhibitor, on VirE2 trafficking. *A. tumefaciens* EHA105virE2::GFP11 cells were infiltrated into transgenic *N. benthamiana* (Nb308A) leaves expressing both GFP1-10 and DsRed. The leaves were infiltrated with 100 μ M ML-7 or 1% DMSO as control 4 h before imaging. The leaf epidermal cells were observed

at 2 d post agroinfiltration under confocal microscope with UPLSAPO $\times 60$ N.A. 1.20 water immersion objective. Red, free DsRed; Green, VirE2-GFP_{comp}. The movement tracking of 20 individual VirE2 aggregates plotted to a common origin is shown below the figure. Scale bar represents 20 μm . (b) Mean velocity of the VirE2 aggregates after ML-7 treatment. (c) Effects of myosin-tail overexpression on VirE2 trafficking. *A. tumefaciens* EHA105virE2::*GFP11* cells harboring a binary plasmid which encodes a tail fragment of respective myosins downstream of 35S promoter were infiltrated into transgenic *N. benthamiana* (Nb308A) leaves expressing both GFP1-10 and DsRed. The leaf epidermal cells were observed at 2 d post agroinfiltration under confocal microscope with UPLSAPO $\times 60$ N.A. 1.20 water immersion objective. Red, free DsRed; Green, VirE2-GFP_{comp}. The movement tracking of 20 individual VirE2 aggregates plotted to a common origin is shown below the figure. Scale bar represents 20 μm . (d) Mean velocity of VirE2 aggregate movement under overexpression of myosin tails. EV, empty vector control. (e) Western analysis of crude extracts from leaf samples agroinfiltrated with myosin-tail constructs. EV, empty vector control. Myosin tails were HA tagged. GFP1-10 was detected to assess the amount of sample loaded, as shown in the lower panel.

Embodiment 5: Fig. 5 illustrated effects of chemicals on respective cell structure

Fig. 5 illustrated effects of chemicals on respective cellular structures. Wild type *N. benthamiana* leaves were agroinfiltrated with binary plasmids encoding different fluorescent markers. The epidermal cells were examined at 2 d post agroinfiltration under confocal microscope with Olympus UPLSAPO 60 $\times$ N.A. 1.20 water immersion objective. Upper panels a-c: leaf samples expressing subcellular markers. Lower panels d-f: leaf samples expressing subcellular markers were treated with chemicals. (a) RFP-MBD. MBD, Microtubule Binding Domain. (b) ER-mCherry. (c) tdTomato-ABD2. ABD, Actin Binding Domain. (d) RFP-MBD. Leaf samples were treated with 500 μM colchicine at 6 h before imaging. (e) ER-mCherry. Leaf samples were treated with 100 $\mu\text{g/mL}$ BFA at 6 h before imaging. (f) tdTomato-ABD. Leaf samples were treated with 20 μM cytochalasin D at 6 h before imaging. Scale bar represents 20 μm .

Embodiment 6: Fig. 6 illustrated that *Agrobacterium*-delivered VirE2 present on the cytosolic side of ER endoplasmic reticulum

Fig. 6 illustrated that *Agrobacterium*-delivered VirE2 is present on the cytosolic side of ER. Wild type *N. benthamiana* leaves were agroinfiltrated with binary plasmids encoding GFP1-10 or GFP11 with or without ER targeting signals. The epidermal cells were examined at 2 d post agroinfiltration under confocal microscope with Olympus UPLSAPO 60 $\times$ N.A. 1.20 water immersion objective. (a) The gene constructs introduced into tobacco cells. (b) Left: co-expression of 2 $\times$ GFP11 and ER-GFP1-10; right: co-expression of ER-2 $\times$ GFP11 and ER-GFP1-10. (c) Left: agroinfiltration of EHA105virE2::*GFP11* harboring a binary plasmid encoding GFP1-10. Right: agroinfiltration of EHA105virE2::*GFP11* harboring a binary plasmid encoding ER-GFP1-10. Scale bar represents 20 μm .

Embodiment 7: Fig. 7 illustrated effects of myosin-tail overexpressions on VirE2 trafficking

Fig. 7 illustrated effects of myosin-tail overexpressions on VirE2 trafficking. *A. tumefaciens* EHA105virE2::GFP11 cells harboring a binary plasmid which encodes a tail fragment of respective myosins downstream of 35S promoter were infiltrated into transgenic *N. benthamiana* (Nb308A) leaves expressing both GFP1-10 and DsRed. The leaf epidermal cells were observed at 2 d post agroinfiltration under confocal microscope with UPLSAPO ×60 N.A. 1.20 water immersion objective. Red, free DsRed; Green, VirE2-GFP_{comp}. (a) Schematic presentations of different myosin-tail constructs that were introduced into tobacco cells. (b) Western analysis of leaf samples agroinfiltrated with myosin tail constructs. EV, empty vector control. Myosin tails were HA tagged. GFP1-10 was detected to assess the amount of sample loaded, as shown in the lower panel. (c) Effects of myosin tail overexpressions on VirE2 trafficking. Z-slices were captured with 0.5 μm step. Extended focus image was shown. Scale bar represents 20 μm. The movement tracking of 20 individual VirE2 aggregates plotted to a common origin is shown below the figure.

Embodiment 8: Fig. 8 illustrated a time-lapse imaging of a NLS1-mutant VirE2 moving on an endoplasmic reticulum strand

Fig. 8 illustrated a time-lapse imaging of a NLS1-mutant VirE2 moving on an endoplasmic reticulum strand. EHA105virE2Δnls1 (amino acids 221KLR. . .KYGRR237 were replaced by alanine) cells were infiltrated into transgenic *N. benthamiana* (Nb308ER) leaves constitutively expressing ER-mCherry and GFP1-10. Relative time is shown at the top right. Scale bar represents 10 μm.

Embodiment 9: Fig. 9 illustrated effects of ML-7 treatment on root tumorigenesis assay

Fig. 9 illustrated effects of ML-7 treatment on root tumorigenesis assay. Root segments from 10-day old wild type *A. thaliana* seedlings were infected with a tumor inducing *Agrobacterium* strain A348. Tumors were photographed three weeks later. (a) Tumors formed with or without ML-7 treatment. ML-7 was added (10 μM) into the col-cultivation mixture and then washed off when the root segments were transferred onto new plates for tumor formation. (b) Tumorigenesis efficiency as determined by percentage of root segments with tumors. (c) Effect of ML-7 on root segment viability. (d) Effect of ML-7 on *Agrobacterium* viability

Embodiment 10: Fig. 10 illustrated effects of RNAi silencing of XI-2 and XI-K on tumor formation

Fig. 10 illustrated effects of RNAi silencing of XI-2 and XI-K on tumor formation. Wild type *N. benthamiana* plants were agroinfiltrated with respective RNAi constructs. The mRNA analysis and agroinfection was conducted three weeks later. (a) Leaf disc tumors with respective genes silenced by RNAi. (b) The mRNA levels of respective genes in silenced plants as detected by real-time PCR. (c) Average fresh weight of leaf discs with tumors.

Embodiment 11: Fig. 11 illustrated an effect of a drug on VirE2 movement

Fig. 11 illustrated an effect of a drug on VirE2 movement. *A. tumefaciens*

EHA105*virE2::GFP11* cell was injected and infiltrated into a transgenic tobacco line Nb308A constantly expressing a GFP1-10 and a DsRed. The drug was injected and infiltrated into a leaf six hours before observation. Two days after the injection and infiltration of *A. tumefaciens*, leaf epidermal cells were placed under a confocal microscope to be observed with a UPLSAPO x60 N.A. 1.20 water immersion lens. The red was a free DsRed; and the green was a VirE2-GFPcomp. A Z-multilayer slice image was acquired with 0.5 μm per step. The diagram in the upper row was a common origin diagram of movement tracks of 20 VirE2 polymers. The diagram in the lower row illustrated an average speed of the 20 VirE2 polymers in movement.

Although the present invention has been described to a certain extent, it is obvious that appropriate changes in various conditions can be made without departing from the spirit and scope of the present invention. It can be understood that the present invention is not limited to the embodiments, but falls within the scope of the claims, which includes the equivalent replacement of each of the factors.

Claims:

1. A method for real-time observation of an effect of a compound or a drug on eukaryotic cells in vitro, wherein the method comprises the following steps of:

1) introducing a marker for observing an effect of a compound or a drug on eukaryotic cells in real time or enabling the eukaryotic cells to contain a marker for observing an effect of a compound or a drug on cells in real time, wherein the marker is an amino acid sequence shown in SEQ No. 1 and SEQ No. 2;

2) adding the compound or the drug into the eukaryotic cells; and

3) observing a morphology, a movement track and/or a speed of the marker in the eukaryotic cells in real time,

wherein the morphology of the marker in the step 3) comprises a size, a shape, a movement mode and/or a position of a filamentous structure formed by a VirE2-GFP;

wherein the observing an effect of a compound or a drug on cells in real time is to observe a movement of a VirE2-GFP on an endoplasmic reticulum/actin network in real time.

2. The method according to claim 1, wherein the eukaryotic cells are selected from plant cells, yeasts, fungi, green algae or animal cells.

3. The method according to claim 1 or 2, wherein the compound is a nucleotide and/or the drug is a small molecule drug.

4. The method according to any one of claims 1 to 3, wherein the compound or the drug is selected from a colchicine, a cytochalasin D, a Brefeldin A, an actin binding domain, a selective myosin light-chain kinase inhibitor ML-7 and a tail fragment of a myosin.

5. The method according to any one of claims 1 to 4, wherein the effect is a nonspecific toxicity of the compound or the drug.