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Distant-Graft Mutagenesis Technology in Soybean

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1. Introduction

As we know, sexual hybridization is the primary means of creating genetic variation in the conventional breeding methods, all kinds of new varieties are mainly developed through this means. However, sexual hybridization can only be carried out between species. The distant-graft mutagenesis technology developed rapidly in recent years just made it possible to bypass the natural obstacles of incompatibility or hybrid embryos sterile of sexual hybridization between distantly-related species and integrate the different sources of rootstock and scion organically, and allow the scion to grow, develop and fruit normally. As the sequence of it, a wide range of mutations can be induced under the adverse conditions of distant grafting and all kinds of the unique new plant types can also be created. Qian (1993), the well-known Chinese scientist, had ever pointed out: "We should develop a technical science---The graft-transformed science of plants, whose role must be no less than genetic engineering."

Compared to other mutagenesis technologies, the distant-graft mutagenesis technology can integrate the advantages of rootstock and scion and further expand the genetic base of target crop. Besides, it characters as: (1) the mutated traits stabilize quickly, which may shorten the breeding period; (2) the operation technique is simple and easy to grasp; (3) the required test conditions and input are comparatively lower than other mutagenesis technologies. Now, the distant-graft mutagenesis technology has been applied in resources innovation and genetic improvement of crops such as wheat, millet, cotton, peanut, mungbean, corn, and soybean etc, and a lot of new plant materials have been created, from which many new varieties with the strong resistance to stress and diseases or the improved yield and quality have been bred out. For example, sweet potato/mungbean, castor/cotton, yam/soybean, sweet potato/soybean and castor/soybean etc are all bred out from the progeny of distant graft. Here we introduce the history, definition, types of distant graft, the procedure and key influencing factors for soybean distant-graft mutagenesis, the types and the possible mechanism of soybean distant-graft mutagenesis and prospect of soybean distant-graft mutagenesis, hoping that this technology can be popularized to widely use in resources

innovation and genetic improvement of soybean and to promote the process of soybean new varieties.

2. History of distant graft

There has been a long history of over 1400 years for application of distant-grafting to agricultural production. The earliest records was found in sixth century encyclopedia of Chinese agricultural knowledge-- "Essential skill to benefit the Qi-people", where it was recorded that the graft union with high survival rate between pearl and mulberry tree yielded the poor quality fruits while the graft union with low survival rate between pearl and jujube or pomegranate tree yielded the good quality fruits. After that, the grafts between different trees, flowers, vegetables and crops were recorded in many agriculture-related books such as "Compendium of Materia Medica" and journals such as "Studies in the History of Natural Sciences" and so forth.

From the purpose of distant graft, it could be found that grafting was originally used to improve plant tolerance to cold, resistance to diseases and enhance quality of fruits in the woody trees cultivation and propagation. Since the discovery of inheritable variation induced by grafting, the distant grafts have been used to innovative plant resources and improve plant varieties. Now, the distant graft technology is not only an effective approach for creating new resources and breeding new varieties, but also an important means in plant physiology, pathology, genetic research.

3. Definition and methods of distant graft

3.1 Definition of distant graft

Broadly speaking, grafting is that the branch or bud or stem of a plant is grafted onto the stem or root of another plant, so that the two parts coming from different plants are connected together and grow into a complete plant. Distant-grafting is that the grafting is carried out among different families/genus/species of plants.

The survived grafted plant, the whole scion and stock form a uniform conductive system, the stems and leaves developed by the scion and the root system of rootstock exchange for nutrient. Because the grafted plant is composed by two different individuals, where the roots of rootstock not only function to absorb water and minerals, but also function to synthesize organic acids and amino acids while the leaves of scion are the main organ for the synthesis of organic nutrients, both two parts are not mechanically combined but are an interdependent organic whole. Rootstock and scion each possess a specific function necessary for survival, and also affect the function of each other, including the synthesis of metabolites and the development of reproductive organs, which is consequently expressed in the progeny of scion seeds.

3.2 Methods of distant graft

There have been many different graft methods developed from the long-term graft practices. According to the resource of scion, the graft methods can be classified into three categories: bud graft, branch graft and stem graft. But according to the grafting modes of stock and scion, the graft methods can be divided into three main categories: plug graft, cleft graft and splice graft. For plug graft, the lower or upper of stem of rootstock seedlings is made a hole by using of bamboo sticks, where the scion is inserted after its stem end is whittled into

wedge shape, so that the rootstock and scion can develop a new plant; cleft grafting is that the scion with wedge-shaped end is inserted the spliced mouth of the stem of rootstock; As for splice graft, all the stems of rootstock and scion are cut into the inclined planes with the same angle and length, and then they are bounded together. Other graft methods such as whip and tongue graft, saddle graft, bridge graft, inarch graft etc. are sometimes used in practices. In addition, according to the combination pattern of rootstock and scion, the graft methods are including single Shoot to root grafts, Y shaped grafts with one scion, interstock grafts, Y shaped grafts with two scions, Y shaped grafts with Y shaped shoots and A shaped grafts with two rootstocks (Jia and Han, 2010).

4. Procedure for soybean distant-graft mutagenesis

4.1 Selection and cultivation of rootstock

4.1.1 Choice of rootstock

The plants with tuber roots are suitable for distant grafting as rootstocks, such as ginger, lily, sweet potato, potato, yam, etc. In addition, other plants with thick stem are also appropriate for grafting as rootstocks, for example, castor, sunflower, sesame, maize, sorghum and so on.

4.1.2 Cultivation of rootstock

The stem node near the surface of soil is often chose for graft site by the way of side-plug when grafting, so it is first assured that stem of rootstock is thick enough to prevent splitting of stem while the scion is inserting the hole of rootstock. In order to increase the stem diameter of rootstock, some necessary methods such as ahead of sowing or cutting off the main stem are generally adopted.

4.2 Appropriate grafting age of rootstocks and soybean scions

4.2.1 The grafting age of rootstocks

Under normal circumstances, the rootstocks are sowed earlier 40-45 days than the scion of soybean. But the climate conditions and the rootstocks types may influence the growth and development of rootstocks, the best age of rootstocks are about 50 days after emergency (DAM) under good growth season.

4.2.2 The grafting age of soybean scions

7-9 DAM soybean seedlings is best for grafting according to our practices, and that seedling age is too small or too big all have important effect on the graft survival rate.

4.3 Grafting operation

4.3.1 Drilling a hole at the stem of rootstock

When grafting, the surface soil near to the stem of rootstock is first scraped away a little with the bamboo blade. Second the nearest internode from the topsoil is decided to use as graft site. Then the surgical blade is sterilized with alcohol cotton and cut off the section of the internode vertically. After that, the sterilized bamboo stick with the wedged end is rotatedly inserted into the cut section at an angle of 30-45 degree along the stem. The size and depth of drilled hole is depended on the stem thickness of scion seedling and the length of wedged end of the scion seedling. Generally, the depth of hole is 1.0-1.5 cm and the diameter of the hole is about 0.2-0.3 cm.

4.3.2 Whittling the stem of scion into wedge end

The robust soybean seedlings are selected as scions. The roots are first rinsed with tap water, and then dried out with the filter paper. After that, the stem of soybean seedling is shaved down to the wedged end from the top root hairs with the wallpaper blade, and the wedge length is generally 1.0-1.5 cm.

4.3.3 Inserting the scion into the rootstock

After pulling out the bamboo stick from the hole of rootstock, the prepared scion is immediately inserted into the hole of rootstock slowly with moderate force, assuring that the scion end just reaches the bottom of the hole.

4.3.4 Embedding the joint site with the clay

After the scion is inserted into the hole of the rootstock, the joint site and scion are immediately embedded and fixed using the clay with a humidity of 60-70%.

4.4 Management measures

4.4.1 Shading the graft union

In order to enhance the survival rate of graft, the graft union must be shaded for about 45 days. The strength of shaded light gradually goes down, and finally drops close to natural condition.

4.4.2 Supplying water for the scion

During the shading period, the rootstock is not only supplied with enough water for normal growth, but also the scion plant is offered necessary water one time per day using the micro-sprayer.

4.4.3 Removing the lateral roots from the scion

7 days after grafting, the lateral roots may grow from the scion. It is necessary to check the newly-grown lateral roots of scion daily, and promptly remove them with a surgical blade. These works will continue to 45 days after grafting so that the scion can host on the rootstocks.

4.4.4 Removing the main stem of rootstock

At 45 days after grafting, the part above the surface soil 10cm of the main stem of the rootstock is cut down.

4.4.5 Later management

After removing the main stem of rootstock, the graft union is transferred to normal growth conditions until the scion plant can mature regularly and the seeds (G0 generation) are gained.

4.5 Identification of mutants

From the G1 generation to G3 generation of grafted scion, there are different types of mutants to take on. The mutated traits include the morphological and physiological traits. Before these mutants are used for genetic research and breeding materials, they had better

be subject to molecular identification through different molecular marker methods. The detailed methods for identification of soybean mutants refer to the methods of Li *et al.* (2003).

5. Main factors influencing distant-graft mutagenesis technology in soybean

Soybean distant-graft mutagenesis technology is originated from the graft practices that enhance resistance to stress or diseases of crops and all the operative steps may influence the survival rate and the mutants-induced rate. Hence, we should pay attention to the key steps when grafting.

5.1 Main factors influencing the survival rate of grafting

5.1.1 Affinity between rootstock and soybean scion

The high or low affinity between rootstock and scion directly affects the survival rate of grafting, so choosing a distant-related plant materials whose affinity with soybean is high will provide an effective safeguarding for distant grafting mutagenesis in soybean.

5.1.2 Viability of rootstock and soybean scion

Viability of the rootstock and soybean scion is critical for success of the graft. Generally, we use plant materials with strong resistance and adaptability as rootstocks and choose soybean varieties with better integrated traits as scion, so that soybean scion can gain enough water and nutrition from rootstock during the process of grafting and the survival rate of grafting is improved.

5.1.3 Methods of graft and level of technology

There are different survival rates of grafting for different graft methods. Though to some extent, selection of graft methods depends on the type of scion and purposes of research, the bud graft is better than the stem graft and branch graft, and the plug is better than the cleft graft and splice graft. For distant grafting in soybean, the side-plug graft on the base of stem is generally an ideal approach. In addition, the skill level of grafting technology has an important impact on the survival rate of graft. If the wedge-shaped stem end of the soybean scion is subject to damage or doesn't get to the bottom of the hole on the base of stem of rootstock when grafting, the seedling of soybean scion would wither to death for lack of sufficient water and nutrient.

5.1.4 The level of management after grafting

Management after grafting can not be ignored. Water supply and shading time must be controlled timely and accurately after grafting. Insufficient shading time and water supply all have an obvious impact on the survival rate of graft. Especially, when checking and removing the lateral roots of scion, the operator do not hurt the root of rootstock.

5.2 Main factors affecting the mutation-induced rate of graft

5.2.1 Combination of rootstock and soybean scion

The grafting combinations between different rootstock and soybean may induce different types of mutant. In our study, it is found that the grafting of different combinations will produce different types of mutations. For example, grafting between soybean and castor often induces seed coat color and oil content mutants in their offspring.

5.2.2 The lateral roots of soybean scion

The newly-grown lateral roots of soybean scion may absorb access water and nutrient from the soil, which will weaken the stress, caused by distant grafting and be not conducive to the induction of mutation. As we noticed, the presence or absence of the lateral roots of scions after grafting is related to whether the mutants occur or not and which generation the mutants occur. When removing the lateral roots of scion in time, the mutated traits can be observed on the grafting generation (G₀). When retaining a lateral root of scion, the emergence of mutated traits may be postponed to the G₁ to G₃ generation. When retaining two or more lateral roots of scion, the grafted progeny may not have any mutants.

5.2.3 The number of retained leaves of soybean scion

The retained leaves numbers of scion when grafting may also have an important impact on the mutation rate. In general, the retained leaves numbers of scion negatively correlate with the mutation rate of grafted progeny. According to our experience, retaining 1-2 leaves of soybean scion is the best option for the early 30 days after grafting.

6. Mutation types induced by distant grafting in soybean

In our research on distant-graft in soybean, we have observed the same morphological mutation as those in mungbean reported by Zhang *et al.* (2002). These mutation consist of the variation of growth habit, growth period, seed size, seed coat color, leaf shape, seed germination capacity and so on, which offer the basic materials for soybean breeding and gene mining. Through the directional selection on the progeny of distant-grafting, we have gained the excellent soybean lines characteristic of good quality, early mature, tolerance to drought and chilling, resistance to stress, lodging and diseases.

The results of karyotype analysis revealed that there was ploidy variation of chromosomes in the offspring of distant grafting in soybean. About 20% of the root tip cell showed chromosomal abnormalities ($2n=36$ or 38) for some individuals in G₁ generation (unpublished). Certainly, there will be other variation such as photosynthetic capacity, isozymes and genes waiting for further research.

7. Possible mechanism of distant-graft mutagenesis in soybean

It is initially thought that the genetic variation induced by grafting is due to integration of genetic material of rootstock into the genome of scion (Taller *et al.*, 1998). However, recent research in molecular biology have identified that though there are indeed great changes for the DNA sequence of scion, the genetic material of rootstock can be detected in the genome of scion. Meanwhile, it is found that the stress-related retrotransposons in the progeny of distant graft have undergone transposition (Xiao, 2005), which as we know is one of the important mechanisms to induce genome rearrangement and gene mutation. In addition, there are also other evidences that the nucleic acid materials of rootstock can be transmitted to the scion through grafting (Stegemann and Bock, 2009), in which the small RNA might silence the special genes or affect the special genes expression of the scion.

Taken our preliminary study and the results of recent studies together, we believe that stock's genetic material is not integrated into the soybean genome, and speculate that distant graft-induced heritable variation in soybean may be the result that the nucleic acid substances of rootstock as a signal molecule is transmitted into the plant of soybean during the distant grafting process, which initiate the special small RNA interfere system and lead

to the genetic mutation, or the stress conditions caused by distant grafting stimulate the stress-related transposable element transposition, leading to the genome rearrangement or gene mutation.

8. Prospects

It is of practically important significance for putting distant-graft technology into innovating excellent soybean materials, broadening genetic base of soybean and promoting breeding of new soybean with good quality, high yield and resistance to stress. However, there is yet no comprehensive system of distant grafting mutagenesis up to now. In particular, the affinity mechanism and the mutation mechanism of distant grafting are not clear, which leads many scholars to disbelief in the distant-graft mutagenesis technology and soybean mutants induced by distant-grafting. Thus, the most important task on distant graft now is to check on genotypes of the known existing soybean lines derived from distant-graft through molecular approach, validating the reliability of applying asexual hybridization (grafting) to soybean breeding. On the basis of it, the affinity mechanism and the mutation mechanism of distant grafting will be exploring, highlighting as soon as possible the true nature of distant-grafting mutagenesis. At the same time, the grafting technology is integrated with the conventional identification and screening approaches of the mutated traits to create soybean germplasm resources with good nitrogen fixation ability, resistance to drought, salt, diseases and aging, and to identify the genetic controlling loci of the related traits. Besides, Grafting technique can be applied in revealing the physiological process mechanisms involved by the signal transduction materials and becomes irreplaceable means to deep understand the interaction of different organs of plant. We believe in the coming future, the distant-grafting mutagenesis technology will be paid more attention by scholars, and play an increasing role on the soybean resources innovation, soybean breeding and gene mining and so forth.

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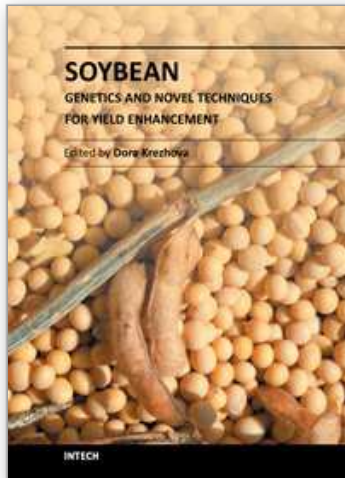
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