Inheritance and Expression of Transgenes through Anther Culture of Transgenic Hot Pepper

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Anther cultures have been developed from transgenic donor peppers carrying HPT/J1-1. Eight out of sixteen plants produced from an anther culture set pepper fruits. Southern blot analysis of donors revealed two independent plants with a single copy of the integrated transgene. PCR and RT-PCR results showed the inheritance of HPT/J1-1 and expression of J1-1 in A_1. All A_1 progeny derived from transgenic anthers had resistance to hygromycin. They grew normally and showed similar phenotypes to the wild-type. Therefore, the use of an anther culture system coupled with genetic transformation in breeding programs will greatly facilitate the genetic improvement of pepper plants.

Key words: Capsicum annuum, T-DNA, Transgenes

Introduction

Pepper (*Capsicum annuum* L.) is one of the most important staple vegetables in the world in terms of its cultivated area and economic value. However, the yield of pepper is unstable due to pests, disease, or injury by successive cropping or unfavourable climate conditions at the harvesting time. Improvements in pepper have been achieved by traditional breeding based on hybridization between species or relative species. For real genetic improvements of pepper, however, new, more sophisticated breeding methods must be developed. Biotechnological advancements in pepper plants to obtain useful traits depend upon the development of efficient and reliable regeneration and genetic transformation methods and progress has been made towards improvements in pepper regeneration and transformation (Zhu *et al*., 1996; Manoharan *et al*., 1998; Lee *et al*., 2004). Haploid or doubled haploid (DH) plants are obtained through anther or microspore culture, and they are currently used for the rapid production of parental lines in F_1 hybrid variety breeding programs for genetic studies and are potential target explants for genetic transformation. These applications might also be of great benefit to pepper breeding methods, especially as more insight into the inheritance of pest and disease resistance will be acquired. For pepper, androgenic embryos or embryogenic calli derived from an anther culture have been successfully induced (Harn *et al*., 1975; Dumas de Vaulx *et al*., 1981) and the anther culture system has been used to produce many DH pepper plants for the use in breeding programs (Chaim *et al*., 2001). The use of the anther culture system to obtain homozygous lines is very simple, cost-effective, and less labour-intensive, and requires less time than other methods. In this study, we report the inheritance and expression of transgenes in the progeny produced from an anther culture of transgenic pepper lines transformed with HPT and J1-1 which is the Pepper Defensin gene.

Results and Discussion

Anther culture response of transgenics

The effect of pretreatment (at 4 and 30 °C) of floral buds on the anther culture response was evaluated with the wild-type (WT). We did not find a beneficial effect of the pretreatment on androgenesis and thus the freshly isolated anthers were directly plated on the callus induction medium (data not shown). However, investigators re-

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Table I. Summary of anther culture of transgenic pepper.

Donor plant	No. of anthers plated	No. of anthers responded (%)[a]	No. of plants regenerated (%)[b]	No. of plants survived[c]	No. of plants set fruits (%)[d]
WT	686	450 (65.6)	277 (61.5)	18	10 (55.6)
J32-2	734	319 (43.4)	47 (14.7)[e]	11	4 (36.4)
J32-3	321	140 (43.6)	33 (23.6)	2	2 (100)
J51-6	465	242 (52.0)	28 (11.6)	2	1 (50)
J51-18	347	214 (61.7)	13 (6.1)	1	1 (100)
Transgenic	1867	915 (49.0)	121 (13.2)	16	8 (50)
Total	2553	1365 (53.5)	398 (29.2)	34	18 (52.9)

[a] (No. of anthers producing calli/No. of anthers plated) × 100.
[b] (No. of plants regenerated/No. of anthers responded) × 100.
[c] Plants transferred to pots in the greenhouse were counted.
[d] Percent of plants bearing seeds in A_0 plants.
[e] For transgenic, plants surviving hygromycin (20 mg l^{-1})-containing medium were counted.

ported that the pretreatment of anthers at low or high temperatures was effective concerning the androgenesis of pepper (Harn *et al*., 1975; Dumas de Vaulx *et al*., 1981).

Callus induction from anthers was higher in the wild-type (65.6%) than in transgenics (49.0%) (Table I). The transgenic plants might undergo more stress due to hygromycin selection, resulting in a slightly lower anther culture response, than the wild-type. The anther culture response among transgenic lines was different, also within a transgenic line or the wild-type, which has the same genetic background. It is known that the response of an anther culture depends upon genotypes of crop species, and differs even within the same genotype (Dumas de Vaulx *et al*., 1981; Kim and Baenziger, 2005). Anther response was visualized within 4–6 weeks of culture, and mainly embryogenic calli were induced. In a regeneration medium containing hygromycin, calli induced from the wild-type anthers were highly susceptible to hygromycin and necrotized, whereas calli from anthers of transgenics were resistant and formed plants.

The rate of plant regeneration was also much higher in the wild-type (61.5%) than in transgenic lines (13.2%). The low frequency of plant regeneration from transgenics might be dramatically decreased by *in vitro* selection pressure, resulting in genome instability. Eighteen plants from the wild-type and 16 plants from transgenics were produced, respectively (Table I). In the pepper fruit setting of A_0 plants, 10 plants from the wild-type and 8 plants from anther cultures of transgenics set seed-bearing pepper fruits. The *in vitro* chromosome doubling spontaneously might occur in pepper plants regenerated from anther cultures. The *in vitro* spontaneous chromosome doubling of anther culture-derived plants has been reported in barley (Hoekstra *et al*., 1993), rice (Cho and Zapata, 1990), and wheat (Kim and Baenziger, 2005).

Seeds (A_1) harvested from anther culture-derived plants were screened on hygromycin-containing medium and the seedlings survived were transferred to soil in a greenhouse. Progeny plants (A_1) grew normally and their phenotypes were similar to those of donor plants. Some of the primary plants (A_0) regenerated from anther cultures showed abnormal phenotypes (data not shown), but the progeny plants (A_1) obtained by self-pollination of A_0 plants revealed normal phenotypes. It is believed that the genome stability of progeny plants might be recovered by self-pollination, although the genetic stability of the progeny plants necessitates further examination.

Inheritance of transgenes in A_1 progeny plants

Genomic DNA of donor plants was digested with *Hin*dIII, and Southern blot analysis with a J1-1 DNA probe showed that the J1-1 gene appeared in the wild-type as well as in transgenics. The reason is that the J1-1 gene was cloned from the pepper plant. In order to verify that wild-type pepper has an intrinsic J1-1 gene, the blot was rehybridized with a DNA fragment of hygromycin phosphotransferase (HPT), showing that only transgenic donors had HPT signals, whereas the wild-type did not reveal any signals. Thus J1-1 exists endogenously in the pepper genome. Southern

blot analysis revealed that a single copy of the transgene was integrated into donor transgenics and that J32-2 and J32-3 were the progeny of J32, and J51-6 and J51-18 of J51. Therefore, the primary transgenic lines used in this study were two independent transgenic events, J32 and J51.

The inheritance of transgenes in the progeny (A_1) derived from an anther culture of transgenics was analyzed by PCR. All the anther culture-derived A_1 plants generated from transgenics contained both J1-1 and HPT, whereas neither of the transgene signals was detected in a plant produced by anther culture of the wild-type. Thus, both transgenes, HPT and J1-1, were transmitted to the progeny (A_1) of anther culture-derived plants.

Expression of transgenes in A_1 progeny plants

Expression of the transgenes was analyzed by RT-PCR on the J1-1 gene and by the hygromycin assay on the HPT gene of an A_1 progeny. RT-PCR results revealed that the mRNA transcript of the J1-1 gene was present in all A_1 progeny plants derived from an anther culture of transgenics, whereas no band was detected in a progeny plant from the wild-type. In hygromycin solution, leaf disc samples of the wild-type and its anther-derived plant turned brown within one week, whereas leaf discs of a progeny from anther-derived transgenics were resistant and remained mostly green, indicating that the HPT gene was functionally expressed in A_1 progeny plants. Therefore, these data indicate that T-DNA-carrying HPT or J1-1 are still functionally expressed in pepper progeny plants (A_1) derived from an anther culture of transgenics.

In conclusion, we demonstrated the stable inheritance of transgenes and successful expression of the transgenes in the progeny produced from an anther culture of transgenic pepper, although further cytological and physiological analyses of these plants are necessary. We anticipate that the use of an anther culture system coupled with genetic transformation in breeding programs will greatly facilitate the genetic improvement of pepper plants in the future.

Experimental

Plant materials and anther culture

The commercial pepper (*Capsicum annuum* L.) cultivar Knockwang was transformed with an *Agrobacterium*-mediated transformation method as described by Ko *et al.* (personal communication). The primary transgenic pepper lines were transformed with pCAMBIA1300::J1-1, which contained the hygromycin HPT gene as a selectable marker and the J1-1 gene encoding an antifungal protein (Meyer *et al.*, 1996). A wild-type (non-transformed) and four transgenic plants (J32-2, J32-3, J51-6 and J51-18) (T_2) were used as donors for an anther culture. Pepper donor seeds were planted in pots in a greenhouse for an anther culture. Anthers were collected from floral buds with 1/4 to 3/4 purple colour (uninucleate stage of the microspore development) by visually determined pigmentation as described by Kim *et al.* (2004). Anthers were plated on MS (Murashige and Skoog, 1962) basal medium supplemented with 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg l^{-1} kinetin and 6% (w/v) sucrose, and then placed in an incubator at 28 °C, in the dark. The induced embryogenic calli were transferred onto regeneration medium containing MS basal medium supplemented with 0.1 mg l^{-1} kinetin and hygromycin at 0 or 20 mg l^{-1} and placed in a growth chamber at 28 °C, with a 16-h photoperiod.

Fertility of anther culture-derived plants

Seeds collected from pepper fruits of anther culture-derived plants (A_0) were plated on MS medium containing 20 mg l^{-1} hygromycin, and plants (A_1) survived were transplanted to pots in the greenhouse. A_0 plants with germinated seeds were classified as fertile if their seeds germinated. Wild-type seeds were germinated on the same medium without hygromycin.

Molecular analysis of plants

Genomic DNA isolation and PCR

Genomic DNA was isolated from young leaf tissues of plants derived from wild-type and transgenic anther cultures according to the method described by Ausubel *et al.* (1997). Two sets of primers were used as follows: for the HPT gene, forward 5′-gaatcttgtcgtgctttcag-3′ and reverse 5′-gtgtcg tccatcacag-3′; for the J1-1 gene, forward 5′-atggctggcttttccaaagtagttg-3′and reverse 5′-tcgtgcagaagcattgaagacggaa-3′. PCR on HPT and J1-1 genes was carried out under the following conditions: 95 °C for 5 min, then 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, and followed by a final cycle at 72 °C for 5 min. Amplified PCR products were electrophoresed in 0.8% (w/v) agarose gel.

Southern blot analysis

Genomic DNA (10 μg) was digested with *Hin*dIII, separated on 0.8% (w/v) agarose gel, and transferred onto a Hybond-N^+ membrane (Amersham Biosciences). J1-1 and HPT probes were generated by PCR with the plasmid pCAMBIA1300::J1-1 as a template. Probe synthesis, hybridization of filters with a probe, and detection were carried out according to the instructions of the manufacturer.

RT-PCR

Total RNA was extracted from leaves of A_1 plants derived from the transgenic and wild-type plants using TRI Reagent (Invitrogen). RT-PCR was performed as described by Kang *et al*. (2002). The first strand cDNA was synthesized from 2 μg of the total RNA in a 20-μl reaction volume using M-MulV Reverse Transcriptase (NEB), and 1 μl of reaction mixture was subjected to PCR in a 50-μl reaction volume. Nucleotide sequences for J1-1 described above and *actin* (forward 5′-aactgggatgatatggagaa-3′ and reverse 5′-cctcaatccagacactgta-3′) were amplified.

Tolerance of plants to hygromycin

To evaluate the hygromycin tolerance in plants derived from the wild-type and transgenics, three leaf discs (6 mm in diameter) from each were dipped in distilled water or 100 mg l^{-1} hygromycin solution, and then incubated at 25 °C under light condition. The tolerance to hygromycin was determined using the difference of leaf discs.

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