Overexpression of Pepper Capsaicinoid Pathway Genes in Tomato

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In this study we attempted to overexpress three pepper genes in tomato to produce spicy fruits. The three genes, BCAT (branched-chain amino acid aminotransferase), Kas (ketoacyl-ACP synthase) and CS/AT (Capsaicin synthase/acyltransferase), were separated by P2Am and T2Am sequences in a tricistronic cassette driven by the 35S promoter. The genes were expressed in transgenic tomato although tomato fruits were not spicy based on two-

Cap aicinoid pa h a gene ; O e e p e ion; Peppe ; Toma o

e p ngenc o hea of ho peppe i he e l of acc m la ion of g o p of alkaloid called cap aicinoid ho e majo ep e en a i e a e cap aicin and dih d ocap aicin [1]. e hea en a ion c ea ed b he e alkaloid i ch de ning a pec of he e c op he gen name Cap ic m come f om G eek kap o, hich mean ‘ o bi e’ [2]. Cap aicinoid a e n he i ed in peppe f i placen a i h in eg a ion of o di e en biochemical pa h a : he phen p opanoid pa h a e phen alanine a b a e o p od ce anill amine, and b anched chain fa acid pa h a hich p od ce 8-me h nonenoic acid ing aline a á b a e [3]. e en me CS (Cap aicin n ha e) combine bo h compo nd o p od ce cap aicin and o he cap aicinoid b conden ing he anill amine i h di e en fa acid b a e [2].

Chilli peppe i an indi pen able pice ed a a ba ic ing edien in a ie ie of c i ine all o e he o ld, he n i ional al e of Cap ic m i high and i con ide ed a an e cellen o ce of i amin C, A, B-comple and E along i h mine al like mol bden m, mangane e, fola e, po a i m, and hiamine [4]. e ape ic p ope ie of Cap ic m a e he e l of cap aicinoid . A a medicine i i main ed a co n e i i an in l mbago, ne algia, he ma ic di o de , and non-alle gic hini i [5]. e plan ha e al o been ed a folk emedie fo d op , colic, dia hea, a hma, a h i i , m cle c amp and oo hache [6]. All he e e ec gge eg la in ake of cap aicinoid i bene cial fo heal h and h ho peppe ho ld be con ide ed a f nc ional food [7].

Unlike o he dome ica ed membe of Solanaceae famil ch a oma o, po a o, and obacco, Cap ic m pecie a e no o io l labo in en i e and di c l o c l i a e. P ngen a ie ie a e o en c l i a ed in open eld and a e lne able o en i onmen al condi ion ha a e de imen al o f i ield [3]. En i onmen al fac o ch a high empe a e, high CO2 le el, and e ce ain can all ed ce he plan g o h/ ield and inc ea e di ea e incidence in he plan [8]. Seed ali and ge mina ion a e a e high dependen on ma i of f i , pecie , c l i a , and po -ha e ing handling [9]. E en if all he ag onomic h dle a e e ol ed, he high a iable cap aicinoid bio n he i hea il con olled b en i onmen ep e en f he p oblem in con i en le el of p ngenc p od c ion in peppe f i [10].

Cla ical b eeding and mode n gene ic manip la ion can bo h be ed fo he imp o emen and con i en p ngenc le el in peppe . B eeding ha con ib ed igni can l o he c ea ion of eli e Cap ic m a ie ie [11]. Ag obac e i m media ed gene ic an fo ma ion a e no o io l ha d fo Cap ic m d e o i ecalci an na e. C en a ailable Cap ic m an fo ma ion p o ocol ha e lo e cienc , poo ep od cibili , and high geno pe-dependence [12,13]. , gene ic manip la ion i no i able op ion fo peppe imp o emen [3]. Enginee ing al e na e model plan o p od ce cap aicinoid co ld be a concep all p omi ing app oach fo p od c ion of he e econda me aboli e [14].

Economicall , oma o (*Solanum lycopersicum*) i he mo impo an ho ic l al c op, and i ield i econd onl o po a o ac o he o ld [15]. De pi e hei clade pli a lea 19 million ea ago [16], genome of Cap ic m and oma o a e ell p e e ed, i h ba ic ch omo ome n mbe of =12 in bo h pecie and majo con e ed n enic egmen be een hem [17]. Toma o being ell-e abli hed model i h highl amenable bio echnological manip la ion me hod and i high p od c i i i h ho c opping c cle [18] can be ed a cap aicinoid biofac o model plan [19]. Ph logene ic anal i of gene familie in ol ed in cap ainoid bio n he i in peppe and hei o holog in oma o, po a o and A abidop i iden i ed 51 gene familie and of he e 13 gene familie had independen peppe peci c d plica ion (ch a ACLd, AT3, b-CT, C3H, CAD, CCR, Ka I and PAL gene) [17]. Compa a i e an c ip ome anal i e ealed e e al gene in cap aicin bio n he i pa h a ha ing di e en e p e ion in peppe and oma o f i . F i peci c e p e ion of CS (encoding ac l an fe a e) p ima il occ ed d ing peppe placen a de elopmen along i h o he gene nece a fo he cap aicinoid bio n he i , con a o hi he o hologo gene in oma o pa h a (BCAT, Ka and CS) a e a el e p e ed in f i ing age [2]. Compa a i e e p e ion die in non -p ngen peppe p ngen peppe al o ho ed la ge dele ion in CS ca e no o e lo e p e ion in non-p ngen peppe ; imila e l e e ob e ed fo o he cap icionoid pa h a gene a ell! e e e l ma indica e ha change in he gene e p e ion of BCAT, Ka and CS/AT enabled cap aicinoid n he i in ho peppe f i [20,21]. M agenic die

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in ho peppe al o e ealed lo of f nc ion in di e en gene a ec he p ngenc di e en l . P ngenc in peppe , he efo e, appea o be nde an c ip ional con ol and i di ec l linked i h highe e p e ion le el of cap aicinoid bio n he i gene in he placen al ep m of p ngen c l i a [21,22]. Compa ed o chilli peppe , in oma o ome gene ha e lo e le el of e p e ion (PAL, C4H, ACL, AMT), o he ha e lo e le el of e p e ion i h empo all e ic ed e p e ion (COMT, FaTA) and fe a e no e p e ed a all (Ka , BCAT and CS) [2].

Ba ed on he gene ic info ma ion on bo h peppe and oma o, i i heo e icall po ible o ac i a e cap aicinoid pa h a in oma o. ee genome enginee ing a egie co ld be ed fo ch endea o . One i o e of an c ip ional ac i a o like e ec o (TALE) o CRISPR/Ca 9 fo m l iple ac i a ion of gene in peppe [23-25]. Second a eg i he e of a ge ed p omo e eplacemen h o gh genome enginee ing fo ac i a ion of inac i e gene in oma o [26,27]. And hi d o ld be in e ion and o e e p e ion of he peppe gene (Ka , BCAT and CS) in oma o h o gh Ag obac e i m media ed an fo ma ion of oma o.

Fo o d e emplo ed Ag obac e i m media ed oma o an fo ma ion o in e and o e e p e he h ee peppe gene in ol ed in cap aicin bio n he i ha e e no e p e ed b oma o, namel BCAT, Ka and CS/AT. T o di e en ec o e e p epa ed one i h 35 p omo e and one i h f i peci c E8 p omo e . All h ee gene e e incl ded in ingle ici onic ec o fo he an fo ma ion (mo e in me hod). All h ee gene a e in ol ed in cap aicin bio n he i h o gh b anched chain fa acid pa h a , BCAT (b anched-chain amino acid amino an fe a e) conden e aline o alpha ke oi o ale a e, Ka (ke oac l-ACP n ha e) along i h ACL (ac l ca ie p o ein) i in ol ed in fa acid n he i h o gh i ob l-CoA o 8-me h l-6-nonenoic acid and CS/AT/PUN1 (Cap aicin n ha e) conden e anill lamine f om phen lp opanoid pa h a i h 8-me h l-6-noneo l-CoA f om b anched chain fa acid pa h a o n he i e cap aicin [2].

pCAMBIA1301 ec o a ed in p epa a ion of o e e p e ion gene con c ! e ici onic ca e e i h h ee peppe gene a a anged a ho n in Fig e 1A! e h ee gene e e epa a ed b P2Ap and T2Ap pep ide (Fig e 1B) f om O bo n MJ [28]! e pep ide e e e e e an la ed o DNA e ence (P2A and T2A in (Fig e 1B) and op imi ed fo plan (A abidop i) ing JCa [29] o p od ce he P2Am and T2Am (Fig e 1B) ed in Fig e 1A! e ca e e (Fig e 1C) a n he i ed b BioBa ic (Amhe , NY, USA) and liga ed in o pCAMBIA1301 a e dige ion i h NcoI and B EII.

Toma o seed (*Solanum lycopersicum*) c Mic o-Tom and Mic o-Tina f om Toma o G o e S ppl Compan , Fo Me e , FL e e face e ili ed in 40 ml of 25% bleach i h 2 d op of een 20 fo 15 min e and in ed 5-7 ime i h di illed a e befo e pla ing in eed ge mina ion medi m (MS al 4.3 g l^{-1}, Ni ch i amin 1 ml l^{-1}, c o e 30 g l^{-1} and aga 6 g l^{-1}, pH 5.8). H poco l and coledon lea e of 7-10 da old eedling e e ed fo an fo ma ion! ee da befo e an fo ma ion, *Agrobacterium* c l e a a ed in 20 ml LB medi m pplemen ed i h 50 mg l^{-1} kanam cin. A da befo e an fo ma ion h poco l and coledon lea e e e c f om eedling a he pe iole and a he ip ing e ile a o on he coc l i a ion media (MS al 4.3 g l^{-1}, hiamine-HCL 0.4 mg l^{-1}, m o-ino i ol 100 mg l^{-1}, c o e 30 g l^{-1}, 2,4-D 0.2 mg l^{-1}, aga 6 g l^{-1} and kine in 0.1 mg l^{-1}) i h e ile l e pape laid on he face. Bac e ia f om LB b o h a ha e ed b cen if ga ion and e pended in coc l i a ion media (i ho aga). Lea e f om o e nigh inc ba ion e e c apped and mi ed i h bac e ia and inc ba ed a oom empe a e fo 30 min e i h occa ional mi ing. Bac e ial pen ion a hen d ained, lea e e e d ied on e ile pape o el and e ned o coc l i a ion media i h l e pape on he face! e e pla e e e ealed i h mic opo e ape and inc ba ed in da k a oom empe a e fo 3 da . Follo ing 3 da of coc l i a ion lea e e e placed on egene a ion medi m (MS al 4.3 g l^{-1}, Ni ch i amin ml l^{-1}, c o e 30 g l^{-1}, ea in 1.5 mg l^{-1}, IAA 0.2 mg l^{-1}, ca benicillin 400 mg l^{-1}, h g om cin 30 mg l^{-1}, aga 6 g l^{-1} and pH 5.8) and inc ba ed nde na al da /ligh c cle fo 3-8 eek i h media change e e 3 eek . Shoo ill egene a e d ing hi pe iod. Regene a ed hoo e e an fe ed o oo ing medi m (MS al 4.3 g l^{-1}, Ni ch i amin 1ml l^{-1}, c o e 30 g l^{-1}, IBA 0.5 mg l^{-1}, ca benicillin 400 mg l^{-1}, h g om cin 30 mg l^{-1}, aga 6 g l^{-1} and pH 5.8) fo f he g o h and oo ing hen an fe ed o oil a e acclima i a ion fo fe da .

T an fo ma ion and in e ion of an gene a con med b plan egene a ion on h g om cin con aining egene a ion media hen b PCR ing h g om cin p ime (fo a d: GATGTTGGCGACCTCGTATT and e e e: GATGTAGGAGGGCGTGGATA) on DNA f om an genic plan .

F om T0 an genic plan , RNA a e ac ed fo RT-PCR. Leaf ample f om o ng an genic plan e e b lked o i ola e RNA. F e h 50-100 mg of leaf i e a f o en in li id N_2 and g inded o po de ing mo a and pe le. One ml of TRI ol eagen a ed o homogeni e 50-100 mg of i e. A e 5 min e of inc ba ion, 0.2 ml of chlo ofo m a added o he ample and f he inc ba ed fo 3 min e ! e mi e a cen if ged a 4 C fo 15 min e ! e pe na an a an fe ed o a ne be and mi ed i h 0.5 ml of i op opanol and inc ba ed fo 10 min e . A e cen if ging a 4 C fo 5 min e , pe na an a di ca ded and RNA pelle a mi ed i h 1 ml of 75% e hanol and cen if ged fo 5 min e a 4 C, pe na an a di ca ded, and pelle e e d ied b in e ing be on clean l e pape fo 10 min e . A o nd 25-30 l of DNa e/RNa e f ee a e a ed o di ol e he RNA. Ali o of hi RNA a ea ed i h DNa e I a 37 C fo 10 min e ! i follo ed phenol: chlo ofo m e ac ion i h i op opanol p ecipi a ion and o 75% e hanol a h! e p i of RNA a ea ed i h DNa e I and an i ed ing Nanod op. Sample i h 260/280 al e be een 1.9-2.1 e e ed fo f he anal i .

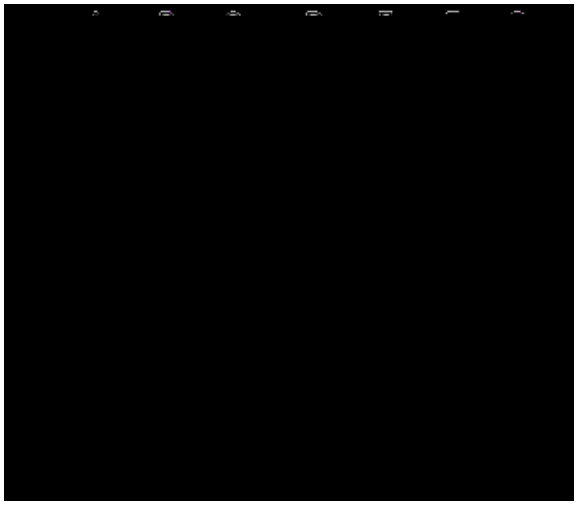
Semi an i a i e RT-PCR a done o con m he an gene e p e ion of ing Sc ip XLT One-S ep RT-PCR ki f om Q an aBio in Applied Bio n he i 2720 e moc cle . Man fac e ' g ideline e e follo ed fo eac ion e p and he moc cle p oced e . In ho , RT-PCR a done fo RNA of bo h an genic and con ol plan in 10 l ol me. All he eagen , RNA and p ime e e ha ed in ice fo 15 min e and he eac ion a e p on ice. Fo each 10 l eac ion, 5 l one- ep To ghMi (20X), 0.2 l each of fo a d and e e e p ime , 0.4 l of Sc ip XLT One-S ep e e e an c ip a e (25X), 2.2 l of n clea e f ee a e and 2 l of RNA e e added and mi ed. PCR pla e a hen p n b ie o emo e an b bble and collec he con en a he bo om of he ell. In he moc cle , RT-PCR a p og ammed a follo : cDNA n he i a 48 C fo 20 min e , ini ial dena a ion 94 C fo 3 min e , 35 c cle of dena a ion 94 C fo 20 econd , annealing 56 C fo 30 econd and e en ion a 72 C fo 1 min e! i a follo ed b 72 C fo 5 min e and 4 C fo fo e e . PCR p od c a n on 1% aga o e gel along i h loading d e o i ali e he p e ence of an gene in RNA.

T an genic oma oe e e eg la checked fo an i al pheno pic change in hei f i hape, face e e on ea f i age o la e ma e age . Ripened oma o f i e e a ed fo picine b o people in lab.

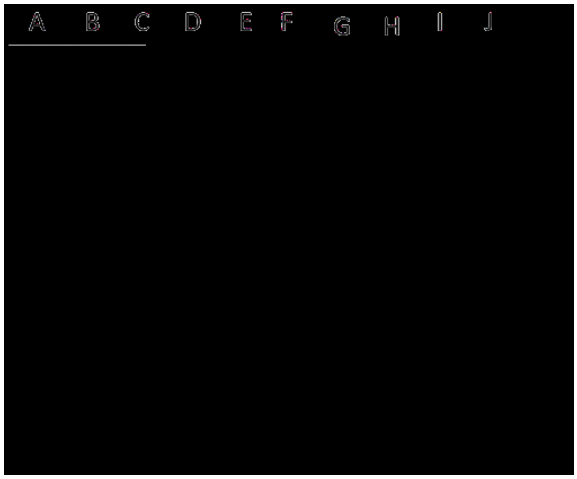
T o oma o line (Mic o-Tina and Mic o-Tom) e e ed fo he an fo ma ion die . Con c i h 35S p omo e a ed o an fo m Mic o-Tina and con c i h E8 p omo e a ed o an fo m Mic o-Tom. Al ho gh he n mbe of infec ed lea e a no co n ed, egene a ion of call f om lea e a a iable fo bo h Mic o-Tina and Mic o-Tom line. Mic o-Tom ho ed ea l ad an age i h be e ge mina ion, fa g o h compa ed o Mic o-Tina (da a no collec ed). D ing call egene a ion ca e a aken o place he lea e ada ial ide p o inc ea e he chance of egene a ion. Ca e a aken no o o e c o d he pla e and e plan e e bc l ed e e 2 eek . Once calli a ed o p od ce he hoo , he e e an fe ed o Magen a bo o gi e eno gh n ien and oom fo plan le o g o fa e . In o al 30 an genic plan f om 35S ec o and 35 an genic line f om E8 ec o e e gene a ed.

Genomic DNA a e ac ed f om he lea e of an genic and con ol oma o plan o pe fo m PCR. H g om cin p ime e e ed o con m he p e ence of an gene in T0 gene a ion of an genic plan (Fig e 2). H g om cin band e e ob e ed in bo h Mic o-Tina and Mic o-Tom an genic line b e e ab en f om con ol plan con ming he in e ion of an gene in oma o genome.

E p e ion of an gene a con med b emi an i a i e RT-PCR ing peppe gene peci c p ime . Fo each p ime e , once he p ime e e de igned, he e e bla ed again oma o e ence o con m hei ab ence f om oma o. Fo each of h ee gene one peci c p ime e a de igned. RT-PCR a done ing he e p ime on RNA de i ed f om con ol a ell a an genic plan . Gel elec opho e i of he RT-PCR p od c ho ed he gene band fo all h ee a ge gene



line 1; C- transgenic line 2; D-Micro-Tina control; E-Micro-Tom control and F-PCR negative control.



Gene expression in transgenic plants. Lanes A-ladder; B, C, D-control for AT, BCAT and Kas gene; E, F, G-transgenic line 1 for AT, BCAT and Kas; H, I, J-transgenic line 2 for AT, BCAT and Kas.

(AT, BCAT and Ka) in bo h an genic line e ed hile he con ol plan did no ha e an an gene e p e ion (Fig e 3). i ho all h ee peppe gene e e in eg a ed in oma o genome and e e being e p e ed.

T an genic plan e e ob e ed fo an pheno pic c al change in plan a ell in f i . e e e e no o e helming change ha e e con i en h o gho all an genic line , ome an genic oma o plan , ho e e , did p od ce f i i h peppe like o gh kin.

Ripened an genic oma o f i e e a ed fo hin of ho ne . T o people a ed he j ice f om he ame f i o de e mine he he he e i an ho ne in i . e e a con en , none of an genic oma o f i ca ied an picine on hem. Al ho gh o e l f om RT-PCR gge ed he e p e ion of all h ee gene in an genic plan , lack of ho ne in f i gge ei he he e i no de ec able inc ea e in cap aicin p od c ion, o he bio n he ic pa h a e i e mo e han 3 gene o be o e e p e ed.

Toma o a a biofac o fo p od c ion of econda me aboli e i no a ne concep , i ha been demon a ed oma o can be ed in ch p po e [30] e e able o e oma o o p od ce be alain (food colo). O he die ha e ed oma oe o inc ea e he p od c ion of a io a onoid [31], phen p opanoid compo nd like e e a ol and geni in [32]. Toma o f i a e ich in me aboli e ch a c o e, he o e , ci a e, mala e and a co bic acid a ell a econda me aboli e ch a ca o enoid , phen p opoid and e penoid i ome e en [33]. e p e ence of ch compo nd gge he p e ence of ba ic bio n he ic pa h a fo each in oma o. e p e ence of he e bio n he ic pa h a ha led man o belie e oma o can be ed a a cha i o p od ce a io economicall a ell a pha macologicall impo an me aboli e ch a e inol (Vi amin A) h o gh B-ca o ene bio n he i [34] and dio cin h o gh e oidal gl coalkaloid p od c ion [35].

e p e ence of def nc cap aicinoid bio n he i pa h a in oma oe o e an oppo ni o p od ce cap aicin in oma oe . i ill alle ia e he p oblem a ocia ed i h peppe fa ming ch a a ing le el of p ngenc , en i onmen al di e , lo eed ge mina ion and long-life c cle a ell a high le el of oil bo ne di ea e and nema ode infec ion [3]. Bo h peppe and oma o being he membe of ame nigh hade famil (Solanaceae) ha e majo con e ed n en i h ba ic ch omo ome n mbe of =12 in bo h pecie and ha e man ai be een hem [18]. Compa a i e genomic e ealed p e ence of all nece a cap aicinoid gene in oma o i h a ing e p e ion [2]. O e e p é ion of ho e le e p e ed gene in oma oe ho ld in heo make oma o f i pic .

B in e ing h ee cap aicinoid gene (BCAT, Ka and AT) e in ended o ac i a e he cap acinoid pa h a in oma o. Ag obac e i m media ed an fo ma ion me hod a cc f l in deli e of all h ee gene a e idenced b egene a ion of h g om cin e i an an genic plan and e p e ion of h ee peppe gene a een in RT-PCR. i , ho e e , did no e l in an inc ea e in cap aicin n he i o acc m la ion in oma o f i . e gene a ion of an genic oma o plan e p e ing peppe gene, ho e e , i a igni can ep o a d he gene a ion of pic oma o! e e co ld be man ea on h o plan e e no p od cing pic f i . I co ld be ha oo li le cap aicin o be de ec ed b a ing, o he h ee gene migh no be eno gh o ac i a e hole cap aicin bio n he i pa h a a i comp i e mo e han 51 gene familie [18]. Gene d plica ion d ing cap ic m e ol ion mean bio n he i pa h a ha p o 13 peppe peci c d plica ion compa ed o oma o [18], al o i e peci c and de elopmen al e p e ion of gene in ol ed in cap aicinoid bio n he i co ld pla ole in making oma o pic .

Wi h pheno pic anal i e did no nd an igni can change be een an genic and con ol oma oe . T an genic oma oe in ome ca e , ho e e , did ha e long inge in ea l age of f i de elopmen and p od ced o gh kin nlike moo h kin of con ol plan . e e cha ac e e e no p e alen . We did no nece a il e pec an genic oma oe o ha e al e ed hape o i e, b he e e e ome in e e ing ob e a ion .

Cap aicin i an impo an econda me aboli e ha ha been a cen al elemen in c lina and pha macological ac i i ie in man co n ie and c l e . e demand fo ch a al able p od c ill likel inc ea e in coming ea . I i ideal e ha e al e na e a of ge ing cap aicin i ho nece a il depending on n eliable and labo -in en i e peppe fa ming. Toma oe being clo e ela i e of

peppe and ha ing def nc cap aicin bio n he ic pa h a co ld be an e a i ha ecen l been a ac ing in e e a a biofac o model fo econda me aboli e .

CSP hank he G ad a e S den O gani a ion (GSO) fo hei ppo . i p ojec a ppo ed in pa b he Uni e i of Lo i iana a Lafa e e . i e ea ch did no ecei e an peci c g an f om f nding agencie in he p blic, comme cial, o no -fo -p o ec o .

CSP pe fo med all he e pe imen and o e he man c ip ; YHW concei ed he p ojec and nali ed he man c ip . All a ho ha e ead and app o ed he man c ip fo bmi ion.

None decla ed.

None decla ed.

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