

Figure S1. Southern blot analysis of T-DNA insertion mutant B7. Genomic DNA was digested with EcoRI or HindIII and hybridized using the hptII gene as the probe. Upper panel, Genetic map of T-DNA. Lower panel, Southern blotting image. M, DNA molecular marker (kb); U, undigested genomic DNA; WT, wild-type strain.



Figure S2. The localization of T-DNA insertion in transformant B7 (upper panel) and the expressions of the two T-DNA flanking genes analyzed by semiquantitative RT-PCR (lower panel). WT, wild-type strain; Hypo, hypothetical protein; gpi, gpi-anchored protein; tub1, tubulin gene.



Figure S3. Pathogenicity assay of *Colletotrichum acutatum* Coll-153 (wild-type, WT) strain and transformant B7 on chili pepper cv. GroupZest. All pepper fruits were inoculated with the WT on the left side and B7 on the right side of a fruit, and lesion sizes were recorded 7 days after drop inoculation.



Figure S4. Schematic illustration of the deletion fragment caused by T-DNA insertion (A) and multiple PCR assays to identify the deleted region in B7 (B). Primer sets 1-12 were used in the PCR assays. Primer set Tub was used to amplify the tubulin gene for the PCR control. The M13r primer was located at the right border region of the T-DNA. Primers Ntp-3'ck and RecQ-3'ck were located downstream of CaNRT2.1 and CaRecQ1, respectively.

7: B7

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	10	20	30	40	50	60	70	80
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Ca>CaNRT2.1	MQLSVLWRAPE	VNPINRKAR	SIPVFNIFNV	ARAFHFSWI	GFMIAFWAWY	TFPPLLTVT	KKDLNLTTAQ	VANSNI
Cn>KXH38191.1	MQLSILWRAPE	VNPINRKAR	SIPVFNIFNV	ARAFHFSWI	GFMIAFWAWY	TFPPLLTVTI	KKDLNLTTAQ	VANSNI
Ch>XP_018163968.1	MQLSVLWRAPE	VNPVNRKAR	SIPVLNIFNV	ARAFHFSWI	GFMIAFWAWY	TFPPLLTVT	KKDLKLTNAQ	VANSNI
Cgr>XP_008090406.1	MQLSVLWRAPE	VNPINRKAR	SIPVFNIFNI	ARAFHFSWI	GFMIAFWAWY	TFPPLLTVTI	KKDLKLNNAQ	VANSNI
Cgl>EQB59180.1	MELSILWRAPE	VNPVNRKAR	SIPVFNVFNV	ARAFHFSWI	GFMIAFWAWY	TFPPLLTVT]	KKDLNLTTAQ	VANSNI
Vd>XP_009649047.1	MKISTLWRAPE	VNPITLKAR	SVPVLNPFNK	GRVFLFSWI	GFMIAFWAWY	TFPPLLTVT]	RKDLALTPQQ	VANSNI
Mo>XP_003710887.1	MGINVKFSDLYRAPE	VNPITRKAR	SIPALNVINM	GRVFFFSWF	GFMIAFWAWY	TFPPLLTVT]	RKDLNLTAAE	VANSNI
Consensus	:::* *:****	***:. ***	*:*.:* :* :	*.*.* ***:	******	********	*:*** *. :	*****
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								1
Ca>CaNRT2.1	VSLVATLFMRLIAGP	ACDRFGSRI	VFGSLLLIGT:	PIGLAPLVK	DATGLYISRE	FIGVLGATEV	/PCQVWCTGFF	DKNVVG
Cn>KXH38191.1	VSLVATLFMRLIAGP	ACDRFGSRI	VFGSLLLIGT:	PIGLAPLVK	DATGLYISRE	FIGVLGATE	VPCQVWCTGFF	DKNVVG
Ch>XP_018163968.1	VSLVATLFMRLIAGP	ACDRFGSRI	VFGSLLLIGT	PIGLAPLVI	NATGLYISRE	FIGVLGATE	/PCQVWCTGFF	DKNVVG
Cgr>XP_008090406.1	VSLVATLFMRLIAGP	ACDRFGSRI	VFGSLLLIGT	LPVGLAPLV1	NVTGLYISRF	FIGVLGATEV	PCQVWCTGFF	DKNVVG
Cgl>EQB59180.1	VSLVATLFMRLIAGP	ACDRFGSRI	VFGSLLLIGS	PIGLAPLVK	DATGLYISRF	FIGVLGATEV	/PCQVWCTGFF	DKNVVG
Vd>XP_009649047.1	VSLSSTLLMRVIAGP	ACDKFGSRW	VFGGMLLLGS	LPIGLAPLVN	SANGLYVSRF	FIGILGGTF	/PCQVWCTGFF	DKNIVG
Mo>XP_003710887.1	VSLVATLFVRMVAGP	LCDLWGSRV	VFGGVLLVGA	PLGLAPLIC	NATGLYVSRF	FIGILGGAF	/PCQVWSTGFF	DKNVVG
Consensus	*** :**::*::***	** :***	***.:**:*:	*:****	***:***	***:**.:**	*****	***:**
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Ca>CaNRT2.1	TANALAGGWGNAGGG	ITYFVMPAV	YDSFVHARGY:	SPGQAWRLTF	IVPVICLFCC	GMGLLLLCEI	DTPTG KWADR H	LHAQEN
Cn>KXH38191.1	TANALAGGWGNAGGG	ITYFVMPAV	YDSFVHARGY:	SPGQAWRLTF	IVPVICLFCC	GMGLLLLCEI	DTPTGKWADRH	LHAQEN
Ch>XP_018163968.1	TANALAGGWGNAGGG	ITYFVMPAV	YDSFVHARGY:	SPGQAWRLTF	IVPVICLFVC	GLGLLLLCEI	DTPTG KWADR H	LHAQEN
Cgr>XP_008090406.1	TANALAGGWGNGGGG	LSYWVMPAV	FDSFVHARGY:	SPGQAWRMTF	IVPVICLLAC	GLGLLLLCK	DTPTGKWADRH	LHAQEN
Cgl>EQB59180.1	TANALAGGWGNAGGG	ITYFVMPAV	YDSFVHARGY:	SPGQAWRLTE	IVPVICLLAC	GLGLLLLCEI	DTPTG KWADR H	LHAQEN
Vd>XP_009649047.1	TANALAGGWGNAGGG	VTYFVMPAV	FDAFVS-RGY	CPGVAWRLTE	IVPLICIITC	GIALLLCDI	DTPTGKWSDRH	LHVQEN
Mo>XP_003710887.1	TANALTGGFGNAGGG	ITYFIMPAV	FDSFVHRMGY	PGQAWRLTE	VVPLVMIIVT	GVSLLLLCPI	DTPTGKWSERH	MHAQQM
Consensus	*****:**:**	::*::****	:*:** **	** ***:**	:**:: ::	*:.***** *	******::**	:*.*:
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	250	260	270	200	290	300	510	320
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Ca>CaNR12.1	L-HSHGVDAAVRSDV	VDVPGGITD	RNNTGSMA	DEEKNS	THGKN	GVVSDHEAS-	-LSRTEMIETA	QADTVV
Cn>KXH38191.1	L-HSHGVDAAVRSDV	VDVPGGITD	RNNTGSMA:	DEEKNS	THGKN	GVVSDHEAS-	-LSRTEMIETA	QADTVV
Ch>XP_018163968.1	L-QSHGVNAV	VDVPGGITD	RTAGSMA	DEEKSS	THGKN	GVIAGNEAP-	LSRTEMVETA	QGDTVV
Cgr>XP_008090406.1	L-RSHGVNAV	IDVPGGIAD	RTIGSMA:	DEEKSS	THGKN	GVIAKNDAP-	-LSGAEMIETA	QGDTVV
Cgl>EQB59180.1	L-QSHGVVDAS-PDV	VDVPGGIAD	RKETAAGSMV:	DEEKRS	NHSSNKNGQH	GVLGDHEVR-	-LSRDEMVETA	RGDTVV
Vd>XP_009649047.1	L-QGHGIQENVLADV	VDVPGGIAD	RHS-TPSPTA	DSEKNS	TNDNKN	ISKPENEAT-	-ISRDEMLVTA	QGETIL
Mo>XP_003710887.1	VGOASTTDATNODKI	VDVPGSITD	KGPNASNSSE	INSEVEEKER	TRK-EKDEQV	GELLDAEAGE	RVIKSDDAAVQ	NTDTIA
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Figure S5. Amino acid sequence alignment of CaNRT2.1 homologs. Cn, *Colletotrichum nymphaeae* SA-01 (KXH38191.1); Ch, *Colletotrichum higginsianum* IMI 349063 (XP_018163968.1); Cgr, *Colletotrichum graminicola* M1.001 (XP_008090406.1); Cgl, *Colletotrichum gloeosporioides* Cg-14 (EQB59180.1); Vd, *Verticillium dahliae* VdLs.17 (XP_009649047.1); Mo, *Magnaporthe oryzae* 70-15 (XP_003710887.1). ID, identity; COV, coverage. ID and COV are displayed in the blast results obtained using BlastP against the NCBI protein database.



Figure S6. PCR screening for CaNRT2.1 gene replacement mutants (A), CaNRT2.1 complementation transformants of the B7 strain and Δ CaNRT2.1 strain F8 (B). A, Schematic representation of gene replacement with three homologous recombination events using the split marker strategy (upper panel) and PCR assays to confirm that three crossover events occurred in potential CaNRT2.1 gene replaced mutants (F8, A1, B1, A5 and A6). MK, DNA molecular marker; P, plasmid carrying the *hptll* cassette as a positive control of hptll crossover and a negative control of 5' and 3' crossover; WT, wild-type strain; N, negative control; tub1 used as the PCR control. Dashed lines indicate the amplified regions of the three crossover fragments. B, PCR amplification of the nptll gene for CaNRT2.1 complementation transformants in B7 and Δ CaNRT2.1 strain F8.

	10	20	30	40	50	60	70	80	90	100	110	120
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Ca-153	MASSAPVTEFITFTFK	NHSTSPPSO	PPAAWSDVTST	LKSVPGVNAI	LYTGTQLEDPS	KTVLVIEWA	SPDAFSAFA	SSEAYMPWFAS	LKAVVA	ASSP	VFYKVPLLSADQ	DPPSV
KXH49087	MASSAPVTEFITFTFK	(NHSTSPPSO	PPAAWSDVTST	LKSVPGVNAI	LYTGTQLEDPS	KTVLVIEWA	SPDAFSAFA	SSEAYMPWFAS	LKAVVA	ASSP	VFYKVPLLSANQ	DPPSV
EXF79771	MASSTPITEFITFK	NHSTSPPSO	PPAAWSDVTST	LKSVPGVNAI	LYTGTQLEDPS	KTVLVIEWA	SPDAFSAFA	SSEAYMPWFAS	LKAVVSAAA	SSSP	VFYKVPLLSANQ	DPPSV
KXH47772	MASSAPVTEFITFTFK	NHSTSPPSO	PPAAWSDVTST	LKSVPGVNAI	LYTGTQLEDPS	KTVLVIEWA	SPDAFSAFA	SSEAYMPWFAS	LKAVVA	ASSP	VFYKVPLLSANQ	DPPSV
XP 01816	MTGPITEFIILTLK	NHATSP-SO	PPAAWSDVTDT	LKSVPGVDAV	VYTGTQIEDPS	KTVLVVQWA	SPEAFSAFA	ASESYMPWFAS	LKAVSAHASI	PDTPGTAPP	LFYKVP-FTADS	DP-AA
XP_00809	MATPITEFIVLTLK	(NHGNSSG	PPAAWSDVTDT	LKSTPGVSAV	VYTGTONEDPS	KTVLVAEWA	SPAAFSEFA	ASESYTPWFAS	LKAVSAHTSI	PDSPGVAPP	LFYKVP-FTADS	NP-AV
CRK19371	MSDPITEFVTFTPF	DLASH	PSAPFGAIAST	LLATPGVESI	LHTGPOHEDPA	THLLAIRWT	SHAAFTAFA	ASDRYTPWLAD	LKALL	GPP	RFHQVRLAHA	AAAAA
Clustal	*: *:***::*:	:	*.*.:. ::.*	* :.***.:	::**.* ***:	. :**:	* **: **	:*: * **:*.	***:	*	*::* *.	
	130	140	150	160	170	180	190	200	210	220	230	240
				••• ••••	.							1
Ca-153	VLHAPCTEVFVAYGV-	-EPEFASKTA	EFAKGLVEGRA	SVAGYHG	HAYGEITTPLA	VDTTSGGGD	GEKGPAVTLI	LLGWDSKOAHL	DAKAKAG-P	ISENIHLLR	SGRKDI	SMYHV
KXH49087	VLRAPCTEVFVAYGV-	-EPEFAGKTA	EFAKGLVEGRA	SVDGYHG	HAYGEISTPLA	VDAVSGGGD	GEKGPAVTLI	LLGWDSKOAHL	DAKAKAG-P	ISDNIHLLR	SGRKDI	SMYHV
EXF79771	VLRAPCTEVFVAYGV-	-EPDFAGKTA	EFAKGLAEGRA	SVAGYHG	HAYGEISTPLA	VDAASGGGD	GEKGPAVTLI	LLGWDNKQAHL	DAKAKAG-PI	ISDNIHLLR	SGRKDI	SMYHV
KXH47772	VLRAPCTEVFVAYGV-	-EPEFASKTA	EFAK GLV E GRA	SVAGYHGI	HAYGEISTPLA	VDTTSGGGD	GEKGPAVTLI	LLGWDSKQAHL	DAKAKAG		-RRKDI	SMYHV
XP_01816	VLGAPCTEVFVAYGV-	-DDSFAGRTA	DFAKGLADGRA	SVEGFHG	HAYGEISTPLA	WEGSGD	GEKGPAVTLI	LLGWDSKQAHL	DAKARPG-PI	ISNNIDLLR	TGRKGI	SMYHV
XP_00809	ALDAPCTAVFIAYGV-	-NDSFLGQTA	EFAKGLTQGGA	SLEGYHGI	HAYGAISTPLA	VAGSGD	GEKGPAVTLI	LLGWDSKQAHL	DAKAKAGRE	YTTSLWSAM	TGDDPTCERAMT	NMSCS
CRK19371	VLTAPCTEIMTAYDIG	RPLFPENLR	LFSERMAAACA	AUGTRGWHAI	NAHGEVTTPIA	REVD	GTPGPAAVL	LIGWDSVEDHA	LAKGGPG-P.	ILDNIELIR	TGRKDV	TLHHY
Clustal	.* **** :: **.:	* .	*:: : *	: *:*.	:*:* ::**:*	*	* ****	*:***. : *	***		*	.:
	250	601/										
	···· ID											
Ca-153	NLKUI 100	100										
KXH49087	NLKUI 92	100										
EXF/9//1	NLKUI 93	100										
KAH4///2	NLKUI 73	98										
VL 01010	NLKKL 75	90										
AP 00809	NIRUISSVEVRP /0	80										
CRR193/1	43	98										
CIUSCAL	****											

Figure S7. Amino acid sequence alignment of CaHP1 homologs. Cs, *Colletotrichum simmondsii* (KXH49087.1); Cf, *Colletotrichum fioriniae* PJ7 (EXF79771.1); Cn, *Colletotrichum nymphaeae* SA-01 (KXH47772.1); Ch, *Colletotrichum higginsianum* IMI 349063 (XP_018163966.1); Cgr, *Colletotrichum graminicola* M1.001 (XP_008090403.1); VI, *Verticillium longisporum* (CRK19371.1). ID, identity; COV, coverage. ID and COV are displayed in the blast results obtained using BlastP against the NCBI protein database.

Signal peptide20	30	40	50	60	70	80 90
Ca MQFKISA-AAFLAFAASALAQH Cn MQFKISA-AAFLAFAASALAQH Cf MQFKISA-AAFLAFAASALAQH Cf MRFTISA-AAFLAFAASALAQH Co MRFTISA-AAFLAFAANVLAQH Cog/MQFKISA-AAFLAFAANVLAQH CgrMRFTTAAVAALVAFVSSAVAQH VI *:*. :* **::**:***	IANFDPVTKPTPNEK IANFDPVTKPTPNEK IANFDPVTKPTPNEQ IANFDPVTKPTPNEK IPNFDPVTKPLSQEK IPNFDPVYKPTSNQK IPDFNPVNKPTPNEK	INAGTSYTIE INAGTSYTIE INAGTSYTIE IDAGSSYTIE IAAGSTYTIE VNAGTSLTIE IPAGSTYKIE : **:: .**	WTAPDAFKDV WTAPDAFKDV WTAPDAFKDV WTAPDAFKDV WTAPDAFKDV WTAPDAFKDV WTAPAKFAGV * ** * *	TVSISLIGGA TVSISLIGGA TVSISLIGGA TVSISLIGGA TVSISLIGGA TVSISLIGGA TIKIALIGGE *:.*:****	TQNTQIPLQDIA: TQNTQIPLQDIA: TQNTQVPLQDIA: TQNTQVPLLDIA: TQNTQVPLLDIA: TQGGQVPLLDIX: TQGGQVPLLDIX:	GIPNSAGKYTW SGIPNSAGKYTW SGIPNSAGKYTW SGIPNSAGKYTW SGVANSAGKYSW SGIPNSAKAYTW SGIPNSALSYEW :*:.*** * *
100 110 Ca TIPSTLGKDAFYGLVVKSESNI Cn TIPSTLGKDAFYGLVVKSESNI Cf TIPSTLGKDAFYGLVVKSESNI Cf TIPSTLGKDAFYGLVVKSEANI Co TIPSTLGKDAFYGLVIKSEANI Cg/AIPSNLGKDAFYGLVIKSEANI CgrKVPADIGGKKFYGLVITSEANI	120 TVDFQYSNPFHIIA TVDFQYSNPFHIIA TVDFQYSNPFHIIA TVDFQYSNPFHIA AADFQYSNPFHIA SVDFQYSNPFHIA AADWQYSNPFVIA	130 GEGSASGTTT GEGSASGTTT GEGAASGTTT SD-SSSGTTT VDGASTGTTT SEGSGSKAPT	140 IVSSSPTATV VVSSSPTATV VVSSSPTATV ITSASGTATV IVSSAGTATV VLATSGTATV YGSGSSTVV	150 TLSAASVSVT TLSAASVSVT TLSAASVSVT TLSAASVSVT TLSAASVSAT TLSATTASVS TTSVGTAIVT	160 PTASASASASASAS PTASASASASASAS PTASASASASASAS PTASASASASASASAS PSASASASASASASAS -AATSAASVSES LSAVPTTSVSTV	170 180 . VTKSESVIV VTKSESVIV VTKSESVIV ASASISKEPVVV ASASSSAPVVIA AIANSSTA ETTTSCPETTTT
VI ::::::::::::::::::::::::::::::::::::	210 PASTITSVTRPATN	220 -STGTATRS- -STGTATRS- -STGTATRS- -ATGTRTPSI IVSGTATSA- TTASVIVQPI	230 ALATVTGVAA ALATVTGVAA ALATVTGVAA TLATVTGVAG -IATVTGSGA ESAPVTVPQN *.**	GA-QATAGVE GA-QATAGVE GA-QATAGVE GA-QATAGVE GA-QATAGVE GA-QATAGVE SA-KGPAGIE GAGQAKAGVE	Sequer AVLGGLAVAALL AVLGGLAVAALL AVLGGLAVAALL AVLGGLAVAALL AVLGGLAVAALL AVLGGLAVAALL AVLGGVALAALL *:**::*:***	ID COV 100 100 99 100 98 100 84 100 70 100 64 100 50 94

Figure S8. Amino acid sequence alignment of CaGpiP1 homologs. Signal peptides and cleavage sequences are indicated with boxes, while the ω site is indicated with an arrow. Ca, CaGpiP1; Cn, *Colletotrichum nymphaeae* SA-01 (KXH47773.1); Cf, *Colletotrichum fioriniae* PJ7 (EXF79770.1); Co, *Colletotrichum orbiculare* MAFF 240422 (TDZ25850.1); Cgl, *Colletotrichum gloeosporioides* Cg-14 (EQB59181.1); Cgr, *Colletotrichum graminicola* M1.001 (XP_008090404.1); VI, *Verticillium longisporum* (CRK19369.1). ID, identity; COV, coverage. ID and COV are displayed in the blast results obtained using BlastP against the NCBI protein database.



(B)



Figure S9. Screening of CaHP1 and CaGpiP1 gene knockout mutants by PCR (A) and Southern blot (B) analysis. (A) Selection marker gene (*hpt*II), 5' and 3' recombination fragments were amplified with primer set P3 and P4, P1 and P2 primers, and P3 and P5, respectively. The full length (FL) of the gene replaced fragment was amplified by primer set P1 and P5. Tubulin1 gene (*tub1*) was used as the positive control in PCR. (B) Southern blot analysis was performed using *CaHP1*, *CaGpiP1* or *hpt*II as the probe. The expected DNA sizes in the Southern bolting assays for the wild type and mutants hybridized with the three probes are indicated below the blotting.



Figure S10. Colony morphology of the *Colletotrichum acutatum* wild-type (WT) strain, CaHP1 mutants (Δ hypo-4a, Δ hypo-3b), CaGpiP1 mutants (Δ gpi-11a, Δ gpi-B79), and transformant B7 on PDA and Czapek's medium containing different nitrogen sources at 5 days postinoculation.



Figure S11. Growth of *Colletotrichum acutatum* Coll-153 strain (WT), CaHP1 mutants ($\Delta hypo$ -4a, $\Delta hypo$ -3b), and CaGpiP1 mutants (Δgpi -11a, Δgpi -B79) under different stresses. A, colony diameter of fungal strains on PDA supplemented with azoxystrobin, tricyclazole or iprodione at 7 days postinoculation.B, colony diameter of fungal strains on PDA amended with NaCl, mannitol, sorbitol, Congo red or SDS at 5 days postinoculation.