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Control of the 1,2-rearrangement process by oxidosqualene cyclases during triterpene biosynthesis†

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Shohei Takase,‡ Yusuke Saga,‡ Nozomi Kurihara, Shingo Naraki, Kenta Kuze, Genki Nakata, Takeshi Araki and Tetsuo Kushiro*

Oxidosqualene cyclases (OSCs) catalyze the cyclization of an acyclic substrate into various polycyclic triterpenes through a series of cation-π cyclization and 1,2-rearrangement processes. The mechanisms by which OSCs control the fate of intermediate carbocation to generate each specific triterpene product have not yet been determined. The formation of ubiquitous sterol precursors in plants, cycloartenol and Cucurbitaceae-specific cucurbitadienol, only differs by the extent of the 1,2-rearrangement of methyl and hydride. In the present study, we identified critical residues in cycloartenol synthase and cucurbitadienol synthase that were primarily responsible for switching product specificities between the two compounds. The mutation of tyrosine 118 to leucine in cycloartenol synthase resulted in the production of cucurbitadienol as a major product, while the mutation of the corresponding residue leucine 125 to tyrosine in cucurbitadienol synthase resulted in the production of parkeol. Our discovery of this "switch" residue will open up future possibilities for the rational engineering of OSCs to produce desired triterpenes.

Introduction

The cyclization of 2,3-oxidosqualene into various cyclic triterpenes represents one of the most complex bio-organic reactions found in nature and has attracted the interest of chemists for decades.[1,2] These triterpenes play a diverse role in biology due to the structural diversity created by oxidosqualene cyclases (OSCs). Moreover, these triterpenes exhibit a wide range of biological activities and are promising leads for pharmaceutical drugs. This enzymatic OSC reaction is certainly the most efficient way to construct complex polycyclic structures from simple acyclic precursors and has been divided into four stages; 1) initiation by the generation of carbocations, 2) series of cation-π cyclizations to produce carbocycles, 3) Wagner-Meerwein 1,2-rearrangement of methyl and hydride, and 4) termination by the quenching of carbocation. OSCs are sophisticated enzymes that precisely control all four steps in order to produce discrete cyclized products from various possible products originating from a common carbocation intermediate. In order to achieve this, OSCs elegantly control the fate of carbocation by regulating the pattern of cyclization and extent of methyl and hydride 1,2-rearrangements. The structural diversity of triterpenes often derives from differences in the extent of methyl and hydride rearrangements. For example, migrated oleanane series such as germanicane, oleanane, taraxerane, multiflorane, glutinane, and friedelin are all derived from differences in the extent of the 1,2-rearrangement process (Fig. S1†).[2] Structural differences in these triterpenes must have resulted from changes in OSCs due to evolution, which slightly altered the active site architecture that influences the extent of 1,2-rearrangements. The so-called "backbone rearrangement" refers to the path by which these rearrangements occur on the triterpene carbon skeleton. Numerous studies on OSCs have revealed the various OSC genes that are responsible for producing many different triterpene products.[2] In addition to OSCs producing a single product, some plant OSCs have

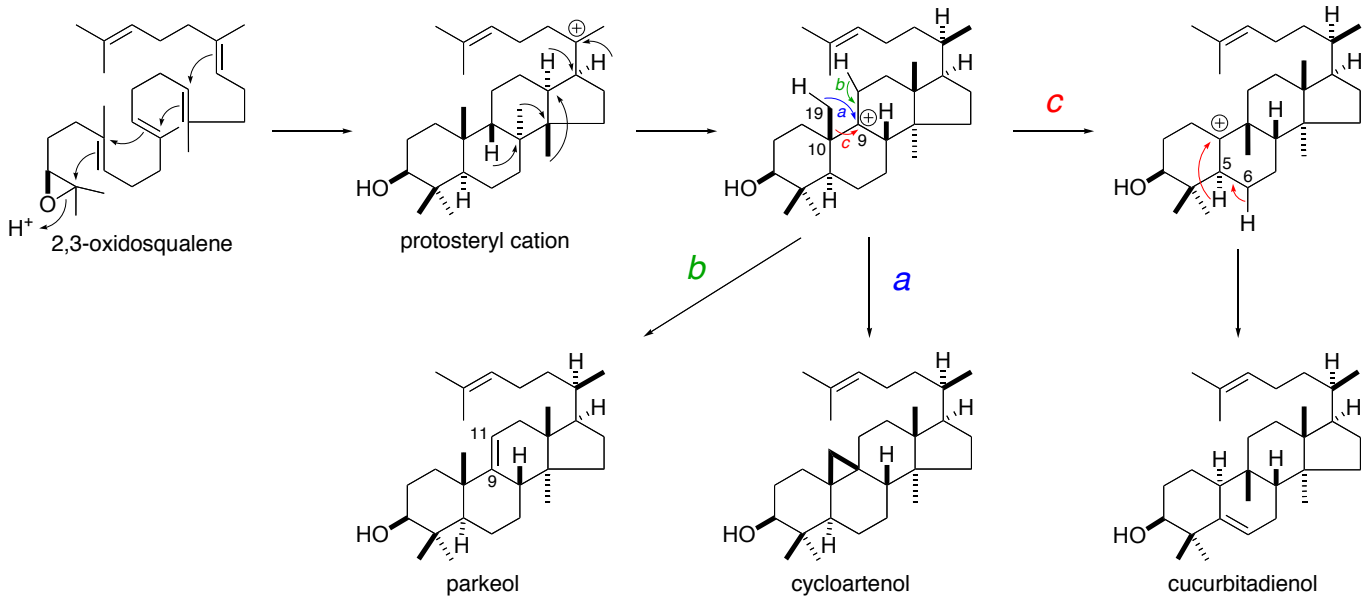

Fig. 1 Cyclization mechanism of 2,3-oxidosqualene into cycloartenol, cucurbitadienol and parkeol.

been shown to produce multiple products within a single active site.[3] While our knowledge on the primary structures of OSCs is increasing, our understanding on the mechanisms by which OSCs control the fate of carbocations according to each reaction path is still limited. To date, only a few studies have been able to identify important residues responsible for governing product specificities.[4]

Cycloartenol is a ubiquitous sterol precursor in plants. It is formed through the cyclization of oxidosqualene, which is folded in a pre-*chair-boat-chair* conformation, to give a tetracyclic protosteryl cation intermediate from which a series of 1,2-rearrangements of methyl and hydride occur. The final deprotonation from the C-19 methyl group at C-9 carbocation stage accompanied by cyclopropyl ring formation produces cycloartenol (Fig. 1, path a). Cycloartenol synthase (CAS) is a dedicated OSC that exclusively produces cycloartenol in this manner.[5] Lanosterol, which serves as a sterol precursor in animals and fungi, is produced in an analogous manner through a nearly identical cyclization scheme, while the final deprotonation takes place at the C-8 carbocation stage to produce a Δ^8 olefinic product. Well-known plant triterpene cucurbitacins are Cucurbitaceae-specific triterpenes that are responsible for the bitter taste of cucumbers, bitter melons, and pumpkins.[6] Cucurbitacins have been shown to exhibit antidiabetic, anti-HIV, and anticancer activities and are important lead compounds for future drug discovery.[6] These cucurbitacins are derived from cucurbitadienol through extensive oxidative modification of the core triterpene structure. Cucurbitadienol is a close structural analog of cycloartenol and its formation differs only in the extent of 1,2-rearrangements and the position of the final deprotonation. Compared to the formation of cycloartenol, an additional C-19 methyl shift from C-10 to C-9 carbocation followed by a hydride shift from C-5 to C-10 and deprotonation from C-6 give rise to cucurbitadienol (Fig. 1, path c). This close analogy in the cyclization mechanism is reflected in the high amino acid sequence identity of up to 80% between CAS and cucurbitadienol synthase (CBS).[7] CBS is also a dedicated OSC that solely produces cucurbitadienol.[7] Considering the ubiquitous role of cycloartenol in plants, it is assumed that CBS originated from CAS in evolution through a number of mutations that altered the 1,2-rearrangement process during the final stage of the reaction cascade. Mutational studies on *Arabidopsis thaliana* CAS revealed that exchanging His477 and Ile481 into the corresponding residues of lanosterol synthase (LAS) nearly completely converted CAS into LAS.[8] These two residues are identical between CAS and CBS, and thus, a different mechanism may be operating in CBS to distinguish it from the formation of cycloartenol. Identifying the residues responsible for the formation of cycloartenol and cucurbitadienol may provide novel insights into the nature of the OSC mechanisms controlling the extent of 1,2-rearrangement processes.

Results and discussion

In order to identify such important residues governing product differentiation between cycloartenol and cucurbitadienol, we first compared the amino acid sequences of both OSCs from several different plants to identify any candidate residues likely to participate in this process. The X-ray crystal structure of human lanosterol synthase (hLAS) was also considered in order to roughly estimate residues located in or near to the active site.[9] Five residues that were conserved among CAS and CBS and characteristically different between the two OSCs were selected, and were Tyr118, Ile365, Pro480, Thr531, and Gly617 of *Pisum sativum* cycloartenol synthase (PSX), which corresponded to Leu125, Leu373, Leu488, Ser539, and Ala625 of *Cucurbita pepo* cucurbitadienol synthase (CPQ) (Fig. S2†).[5,7] All of these residues were likely to be located in

or around the active site. According to the crystal structure of hLAS complexed with the product lanosterol,[9] Tyr118 of PSX corresponded to Tyr98 of hLAS, which was located close to the B-ring of lanosterol and appeared to be in an ideal position to interfere with the 1,2-rearrangement process discriminating between the formation of cycloartenol and cucurbitadienol (Fig. S3†). Pro480 of PSX also corresponded to Ile452 of hLAS, which was located upstream of Val453, a residue that has been shown to strongly influence the product outcome between lanosterol and cycloartenol.[8] Thus, differences in this residue between CAS and CBS may be responsible for product specificity. Gly617 of PSX was located next to the Tyr residue strictly conserved among all OSCs and corresponded to Tyr587 of hLAS, which was oriented toward the A ring of lanosterol. Any difference in the Gly617 position may affect the positioning of the conserved Tyr residue, which may, in turn, influence the conformation of the substrate or an intermediate. Thr531 of PSX was also located next to a conserved Tyr residue among CAS, CBS, and LAS, and corresponded to Tyr503 of hLAS, which was located above the B ring of lanosterol and has been proposed to be involved in the final deprotonation during the formation of lanosterol. Ile365 of PSX corresponded to Ile335 of hLAS, which was located adjacent to Tyr503 in space, and, therefore, may influence the positioning of the important Tyr residue and alter the position of the final deprotonation from an intermediate carbocation. All other residues that were predicted to be located within the active site based on sequence comparisons with hLAS were all conserved among CAS and CBS, thereby reflecting the close relationship of cyclization mechanisms between the two enzymes.

Single point mutants were prepared for PSX and CPQ for each of the five residues discussed above. Mutations were made to exchange residues between the two OSCs. The resulting mutants were PSX Y118L, PSX I365L, PSX P480L, PSX T531S, PSX G617A, CPQ L125Y, CPQ L373I, CPQ L488P, CPQ S539T, and CPQ A625G. All mutant cDNAs were prepared by PCR, sequence checked, ligated into the yeast expression plasmid pYES2, and expressed in the *Saccharomyces cerevisiae* mutant strain GIL77, which lacked

Mutants	cycloartenol	cucurbitadienol	parkeol
PSX Y118L	Δ	O	–
PSX I365L	O	–	–
PSX P480L	O	–	–
PSX T531S	O	–	–
PSX G617A	O	Δ	–
CPQ L125Y	–	Δ	O
CPQ L373I	–	Δ	–
CPQ L488P	–	O	–
CPQ S539T	–	–	–
CPQ A625G	–	O	–
CPQ 3-mut	–	–	O
CPQ 13-mut	–	–	Δ

Table 1 Products identified from each mutant prepared. "O" represents a significant while "Δ" represents a minor production of the compound. "–" represents not detected.

internal LAS activity.[10] Cells were harvested and extracted with hexane and analyzed by TLC and GC-MS. Among the five mutants from PSX, the Y118L mutant gave a major product spot on TLC, the R_f value of which was different from that of cycloartenol and identical to that of cucurbitadienol (Fig. S4 and S5†). A minor product spot was also observed that corresponded to cycloartenol. The G617A mutant also showed a faint spot that corresponded to cucurbitadienol. All other mutants gave an identical TLC pattern with wild-type PSX (Table 1). A GC-MS analysis clearly showed that the major product produced by the Y118L mutant corresponded to cucurbitadienol while the minor product corresponded to cycloartenol (Fig. 2 and S6†). In order to fully confirm the production of cucurbitadienol by this mutant, a large scale culture (1 L) was prepared and samples were extracted with hexane and purified by silica gel column chromatography. The ^{1}H-NMR spectrum of the cucurbitadienol fraction exhibited 8 methyl signals at δ 0.80 (C30), 0.84 (C18), 0.90 (d, C21), 0.91 (C19), 1.02 (C29), 1.13 (C28), 1.60 (C27), and 1.68 (C26) (including one secondary methyl and two vinylic methyls), a hydroxymethine signal at δ 3.47 (C3), and two olefinic signals at δ 5.09 (C24) and 5.59 (C6), all of which completely agreed with that of authentic cucurbitadienol (Fig. S7 and S8†).[7] Therefore, the PSX Y118L mutant produced cucurbitadienol as a major product while retaining the ability to produce only minor amounts of cycloartenol.

On the other hand, of the five mutants of CPQ, only the L125Y mutant showed a different product pattern on TLC, giving a product spot that corresponded to a typical triterpene monoalcohol while a faint spot for cucurbitadienol remained (Fig. S9†). The S539T mutant completely abolished activity, whereas the L488P mutant retained wild-type activity (Table 1). A GC-MS analysis of the product from the L125Y sample indicated that the major product peak was slightly different from cycloartenol in its retention time while only a small amount of cucurbitadienol was visible (Fig. 3). In order to identify the major triterpene product, a sample was prepared from a large scale culture (1 L) and analyzed by ^{1}H-NMR. Although spectral resolution was not high due to limited amounts of the sample, 7 methyl signals (out of 8) at δ 0.65, 0.74, 0.82, 0.99, 1.04, 1.60, and 1.68, a hydroxymethine signal at δ 3.23, and two olefinic signals at δ 5.09 and 5.21 were clearly observed that corroborated well with the previously reported value of parkeol (Fig. S11†).[11] Since parkeol has been observed in many of the LAS mutants

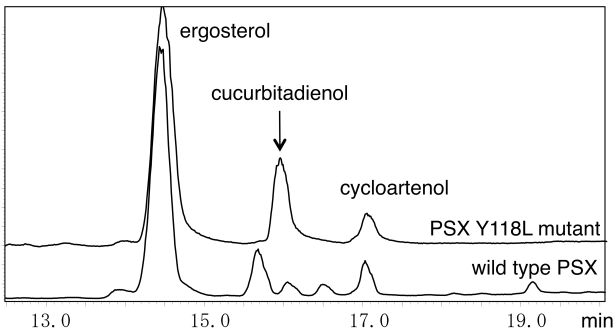

Fig. 2 GC-MS analysis of the hexane extract from the PSX Y118L mutant.

reported previously and its production by the CPQ mutant is a reasonable consequence, we concluded that the CPQ L125Y mutant almost exclusively produced parkeol while a very minor amount of cucurbitadienol remained. Taken together, these results demonstrated that Tyr118 of PSX and the corresponding Leu125 of CPQ were critical for product specificity and that this residue almost exclusively controlled the 1,2-rearrangement process during the final stage of the cyclization reaction. To the best of our knowledge, this is the first study to identify such a critical residue in OSC that controls the rearrangement process. The results from the PSX Y118L mutant revealed that with a single mutation, CAS was changed into nearly perfect CBS producing 75% of the product as cucurbitadienol. It was unexpected that CAS already harbored the potential ability to produce cucurbitadienol with only a single amino acid mutation. Thus, it may had been a minimal task for evolution to identify and mutate this critical residue of the housekeeping CAS in order to allow it to produce the different metabolite cucurbitadienol, which became a characteristic triterpene among Cucurbitaceae plants. It was also unexpected that plants other than Cucurbitaceae did not evolve to produce cucurbitadienol as it appears to be easy to convert CAS into CBS with only a single mutation. The exact cause of the changes in product specificities with the Tyr to Leu mutation and vice versa on each enzyme are still speculative; however, since the major difference between the two residues are their sizes, differences in the size of the residue at this position may affect the 1,2-rearrangement process. The large Tyr residue in CAS may preclude further rearrangements of the C-19 methyl group from C-10 to C-9 based on steric hindrance while this process is possible with the smaller Leu side chain in CBS. Some electronic effects such as cation-π stabilization from the Tyr residue with intermediate carbocation may also be possible; however, this effect is not possible with the Leu residue in CBS and it is difficult to explain why the absence of such stabilization resulted in an extended rearrangement process. Alternatively, mutations may affect the electrostatic environment of the active site, which may, in turn, alter the relative energies of intermediate carbocations that lead to a kinetically favored product, as has been proposed in calculation studies on a related case.[12] Further mutational studies on this residue are needed in order to gain further insights into the actual cause of the difference in the 1,2-rearrangement reaction.

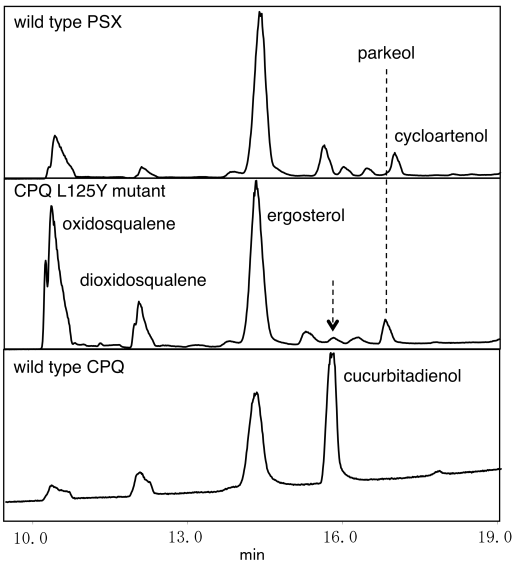

Fig. 3 GC-MS analysis of the hexane extract from PSX (top), the CPQ L125Y mutant (middle), and CPQ (bottom). The dotted arrow in the middle panel indicates the minor amount of cucurbitadienol produced.

The CPQ L125Y mutant produced parkeol, but not cycloartanol, which prompted us to consider the existence of a factor necessary to produce cycloartenol. The Leu to Tyr mutation on CBS was successful to preclude the 1,2-rearrangement process at the C-9 carbocation stage; however, it failed to produce the cyclopropyl ring by deprotonation from the C-19 methyl group. In CAS, none of the residues have been proposed to play a role in the final deprotonation from the C-19 methyl group. Furthermore, the underlying mechanism of unusual cyclopropyl ring formation in the biosynthesis of any of terpene, such as in pre-squalene pyrophosphate formation, has not yet been elucidated in detail.[13] While the emergence of the production of lanosterol was observed by many of the CAS mutants, that of cycloartenol by LAS mutants was almost impossible in the past. There has been only one study on a LAS mutant from a plant that produced minor amounts of cycloartenol.[14] In this case, parkeol was produced as a major product. In an effort to allow the CBS mutant to produce cycloartenol, we introduced mutations at residues that presumably formed the active site and were also located within second-sphere residues so that all of these residues become identical to CAS (Fig. S12 and S13†). To achieve this, 13 mutations were introduced simultaneously into CPQ including L125Y, and were S123G, L125Y, L129M, L228M, L373I, L488P, L527I, S539T, T574A, M575I, E576Q, C617S, and A625G (Fig. S12†). Therefore, this mutant (CPQ 13-mut) was predicted to possess an identical active site with CAS, at least up to second-sphere residues (Fig. S13†). We also prepared the CPQ mutant with 3 mutations around Leu125 because this residue was critical for product differentiation; therefore, residues nearby may have strongly influenced the formation of cycloartenol. This mutant (CPQ 3-mut) included S123G, L125Y, and L129M. These mutants were expressed in yeast GIL77 and their products analyzed by TLC and GC-MS. The results obtained showed that, in CPQ 13-mut, only a faint product spot was visible on TLC and corresponded to triterpene monoalcohol, and a GC-MS analysis showed that this product was parkeol, not cycloartenol (Fig. S14 and S15†). Similarly, CPQ 3-mut only produced parkeol, however, the production level of parkeol was nearly identical with that by CPQ L125Y mutant. Therefore, even with 13 mutations that presumably changed all of the residues in and around the active site to make it identical with CAS, the production of cycloartenol was still not detected. This result suggested that some other residues distantly located from the active site strongly influenced the production of cycloartenol. As has been proposed, product specificity during 1,2-rearrangements is likely to be controlled by kinetics, and, therefore, the formation of cycloartenol may require a basic residue responsible for deprotonation from the C-19 methyl group.[12] The effects of distant residues may alter the electrostatic environment within the active site,

thereby favoring the formation of cycloartenol over that of parkeol. Not only mutational studies, but also structural studies and probing dynamic motions of CAS are needed to obtain a deeper understanding of the nature of the formation of the characteristic cyclopropyl ring during the biosynthesis of cycloartenol.

Conclusions

Mutational studies on triterpene synthase CAS and CBS have revealed for the first time that a critical residue plays a major role in controlling the 1,2-rearrangement process during a sophisticated enzymatic oxidosqualene cyclization reaction. The mutation on Tyr118 of CAS into Leu conferred the ability to produce cucurbitadienol, a significant gain-of-function mutation that converted CAS into nearly perfect CBS. The reverse mutation of Leu125 into Tyr in CBS resulted in the preclusion of 1,2-rearrangements at the C-9 carbocation stage and production of parkeol. The steric size of the active site residue was suggested as a factor controlling the 1,2-rearrangement process. Alternatively, mutations may affect the electrostatic environment of the active site, resulting in a kinetically favored product. Further multiple mutations around the active site on CBS that presumably harbors the identical active site with CAS failed to produce cycloartenol, suggesting that some distant residues strongly influenced the formation of the cyclopropyl ring in the biosynthesis of cycloartenol. It is tempting to speculate that a single residue may be in charge of controlling the extent of the 1,2-rearrangement process in other cases such as in migrated oleanane series. The identification of such a 'switch' residue will be an important step towards the rational engineering of OSC to produce desired triterpenes.

Experimental section

Construction of mutant cDNAs

Site-directed mutagenesis on PSX and CPQ was carried out using PCR as described previously.[15] Briefly, the mutation primers listed in Table S1 were designed and used for 1st PCR with either N- or C-terminal primers harboring appropriate restriction sites with wild-type PSX or CPQ cDNA as a template. PCR was carried out with Phusion DNA polymerase (NEB) for 30 cycles with a program (98˚C, 10 sec, 58˚C, 30 sec, 72˚C, 20 sec, and final extension at 72˚C, 10 min). The resulting amplified fragments were gel purified and used as primers together with the remaining C- or N-terminal primers for 2nd PCR with a program (98˚C, 10 sec, 58˚C, 30 sec, 72˚C, 80 sec, and final extension at 72˚C, 10 min). The amplified 2.3 kb fragments corresponding to full length cDNA were digested with restriction enzymes and ligated into the yeast expression vector pYES2 (Invitrogen) under the GAL1 promoter and sequenced to confirm that only the desired mutation had been introduced.

Functional expression in yeast

The resulting plasmids were introduced into the yeast strain GIL77 using a Frozen EZ yeast transformation kit (Zymogen), plated onto a SC-U plate supplemented with ergosterol (20 μg/mL), hemin (13 μg/mL), and Tween 80 (5 mg/mL), and cultured at 30˚C for selection. The resulting colonies were cultured in liquid SC-U medium supplemented with ergosterol, hemin, and Tween 80. The expression of proteins and product isolation were carried out as described previously.[10] The extract was applied to a silica gel TLC plate (Merck 60 F_{254}, 0.25-mm thickness), developed with benzene/acetone (19:1), stained with phosphomolybdic acid (5% ethanolic solution), and heated.

Product analysis

A GC-MS analysis was carried out with Shimadzu QP2010SE with an Rtx-5MS column (250 μm i.d. x 30 m, film thickness of 0.25 μm, Restek) with a helium carrier (flow rate 1 ml/min). The column temperature was raised from 250˚C to 320˚C at a rate of 15˚C/min and maintained at 320˚C for 12.5 min. The injection temperature was 250˚C, electron ionization 70 eV, ion source temperature 250˚C, and mass scan range for *m/z* 45–600 with a 0.2-sec collection interval. ^{1}H-NMR spectra were measured on a JEOL JNM-AL300 spectrometer (300 MHz) with $CDCl_3$ (99.8% atom ^{2}H, Kanto Chemical) as a solvent.

Construction of CPQ 13-mut and 3-mut clones

Multiple site-directed mutageneses were carried out on wild-type CPQ using the mutation primers listed in Table S3. The CPQ-1,2,3-mut-A primer contained S123G, L125Y, and L129M mutations. The CPQ-4-mut-A primer contained the L228M mutation. The CPQ-L373I-A primer contained the L373I mutation. The CPQ-L488P-S primer contained the L488P mutation. The CPQ-7,8-mut-S primer contained the L527I and S539T mutations. The CPQ-9,10,11-mut-S primer contained the T574A, M575I, and E576Q mutations. The CPQ-12,13-mut-S primer contained the C617S and A625G mutations. PCR was initially carried out using N-terminal and CPQ-1,2,3-mut-A primers with wild-type CPQ cDNA as a template to amplify a fragment harboring the first three mutations. After gel purification, this fragment was used as a primer together with the CPQ-4-mut-A primer to amplify a fragment that contained the 4th mutation. PCR was carried out again using this purified fragment and the CPQ-L373I-A primer to amplify a fragment that contained the 5th mutation. A PCR fragment containing the 6th through 13th mutations was independently prepared using CPQ-L488P-S, CPQ-7,8-mut-S, CPQ-9,10,11-mut-S, CPQ-12,13-mut-S, and C-terminal primers by repeated PCR. These two fragments from the N- and C-terminal halves were then used as primers with wild-type CPQ cDNA as a template to amplify the full length fragment of CPQ 13-mut and the sequence obtained was checked. CPQ 3-mut was similarly prepared using the CPQ-1,2,3-mut-A primer with the N- and C-terminal primers.

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School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan, E-mail: kushiro@meiji.ac.jp; Tel: +81-44-934-7105

‡ These authors contributed equally to this work.

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